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USE OF *TRICHODERMA HAMATUM* FOR BIOCONTROL OF LENTIL VASCULAR WILT DISEASE: EFFICACY, MECHANISMS OF INTERACTION AND FUTURE PROSPECTS

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Abstract: *Trichoderma hamatum* (Bonord.) Bainier was evaluated for its antagonistic potential against *Fusarium oxysporum* Schlecht. emend. Snyder and Hansen sp. *lentis*, the causal agent of vascular wilt disease of lentil (*Lens culinaris* Medikus). Hyphal interactions on Petri plates resulted in an increase in the number of conidial spores and an increase in the vegetative growth of *T. hamatum,* and a decrease in the hyphal formation and sporulation of *F. oxysporum* f. sp. *lentis*. Electron and light microscopical observations suggested that hyphae of *T. hamatum* established aggressive contact and attachment with the hyphae of the pathogen. Growing in parallel, coiled densely and tightly, *T. hamatum* may penetrate those of the pathogen hyphae causing collapse due to the loss of turgor pressure. The cellulolytic enzymes produced by *T. hamatum* presented sufficient characteristics for its antifungal activity in the hyphae hydrolysis and competition process. In growth room and glasshouse experiments, the addition of the conidial suspension of *T. hamatum* or its culture filtrate to soil, significantly ($p \leq 0.05$) reduced development and spore germination of *F. oxysporum*. In the rhizosphere, *T. hamatum* occupied the same ecological niches (rhizosphere, roots, and stems) parasitizing *F. oxysporum* f. sp. *lentis*. Treatments using *T. hamatum* delayed the time of infection by *F. oxysporum*, promoted the growth, and increased the dry weight of a susceptible variety of lentil (cv. Precoz). The percent of mortality was reduced to 33 and 40% when using *T. hamatum* and its filtrate, respectively, compared to 93% in the control treatment during the 65 days of growing in loam/sand (2:1 vol/vol) under glasshouse conditions. Plant colonization results indicate that *T. hamatum* and its filtrate significantly ($p \leq 0.05$) reduced development of the pathogen in the vascular tissue of lentil to < 30 and < 40% stem colonization, respectively, compared to 100% in the plant pathogen control. Our results suggest that potential biocontrol mechanisms of *T. hamatum* towards *F. oxysporum* f. sp. *lentis* were antibiosis by production of antifungal enzymes, complex mechanisms of mycoparasitism, competition for key nutrients and/or ecological niches, growth promotion, and a combination of these effects. This study results hold important suggestions for further development of effective strategies of the biological control of Fusarium vascular wilt of lentil.

Key words: *Fusarium oxysporum* f. sp. *lentis*, mycoparasitism, rhizosphere populations, soil treatment, *Trichoderma hamatum*

INTRODUCTION

Vascular wilt is one of the most economically important fungal diseases in many lentil-growing regions of Syria and worldwide (Saxena 1993; Bayaa and Erskine 1998; Erskine *et al*. 2009) and is caused by *Fusarium oxysporum* Schlecht. emend. Snyder and Hansen f. sp. *lentis* Vasudeva and Srinivasan (1952). This wilt pathogen survives in the soil as chlamydospores that can remain viable for several years (Erskine and Bayaa 1996) and is capable of colonizing residues and roots of most crops grown in rotation with lentil. The incidence of the wilt disease is increasing, causing substantial lentil yield losses. Yield losses higher than 70% have been reported in Syria (Bayaa *et al*. 1986). The use of broad-spectrum fungicides further results imbalances in the microbial community. These imbalances create unfavourable conditions for the activity of beneficial organisms. Broad-spectrum fungicides also cause environmental pollution as well as detrimental effects on human health. Biological control of *Fusarium* wilt diseases has been demonstrated in some cases and represents an additional tool that may provide effective and sustainable disease management. The practice of relying less on chemical inputs reflects consumer concerns over pesticide residues. Biological control has become an important aspect of sustainable agriculture (Cook and Baker 1983; Baker and Paulitz 1996) and food production.

Trichoderma species are typically known to be soilborne, green-spored ascomycetes that can be associated with the roots of plants as well as in the rhizosphere. The *Trichoderma* species are commonly considered a key genus in agricultural soils. These species are known for their potential to control plant disease in what can be a close association with many aspects typical of endophytic associations for plant health and growth (Harman

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et al. 2004; Berg *et al.* 2005; Bailey *et al.* 2008; Bennett and Whipps 2008). *Trichoderma* spp. are the most common mycoparasitic and saprophytic fungi. They are highly successful colonizers of their habitats and attack a great variety of phytopathogenic fungi. Such fungi are responsible for important diseases of major economic crops worldwide (Bastos 1996; O'Neill 1996; Samuels *et al.* 2000; Brozová 2004; Harman *et al.* 2004; Vinale *et al.* 2006; Bailey *et al.* 2008). Furthermore, a considerable number of studies revealed that *Trichoderma* can inhibit plant pathogens by producing secondary metabolites such as antibiotics (Sivasithamparam and Ghisalberti 1998; Howell 2003) and cell wall-degrading enzymes (Lorito 1998; Elad 2000) such as chitinases (Benhamou *et al.* 1994; Metcalf and Wilson 2001), β-1,3-glucanases (Lorito *et al.* 1994; El-Katatny *et al.* 2001), cellulases (Kovács *et al.* 2009; De Castro *et al.* 2010), proteases (Haran *et al.* 1996) and other hydrolases (Prasad *et al.* 2002).

In our evaluation studies, *Trichoderma hamatum* (IMI388876) was selected from a large number of bacterial and fungal organisms as the most active and antagonistic isolate to use in the biological control of Fusarium vascular wilt on lentil (El-Hassan 2004). The principle objectives of the current research were to: (i) understand the efficacy of *T. hamatum* and its culture filtrate as a means to develop an effective biological control agent for *F. oxysporum*, (ii) study the production of fungal cell wall-degrading enzymes and understanding the mode of hyphal interactions of *T. hamatum* on *F. oxysporum* f. sp. *lentis* and (iii) outline the competitive success as well as monitor the relationship between antagonist and pathogen populations in the rhizosphere, roots and stem after application, noting their impact on disease severity development and wilt incidence on lentil plants.

MATERIALS AND METHODS

Fungal antagonist and pathogen cultures

T. hamatum (Bonord.) Bainier (IMI388876) was isolated from rhizosphere of a lentil crop in Syria using the soil dilution plating technique. A mineral agar-based *Trichoderma* selective medium (TSM) (Askew and Laing 1993) was developed by El-Hassan (2004) and used to reisolate and enumerate *T. hamatum* from soil, root and plant materials. The composition of TSM was as follows: (g/l):0.2 $MgSO_4.7H_2O$, 0.9 K_2HPO_4, 0.15KCl, 1.0 NH_4NO_3, 3.0 D(+) glucose, 0.15 rose-bengal and 20.0 agar (Oxide, Basingstoke, UK) in 1 litre of sterile distilled water (SDW). Following autoclaving, 0.1 mg Chloramphenicol, 0.1 mg PCNB, 0.05 g Captan, and 0.32 ml Metalaxyl were added to a basal medium before pouring in the plastic Petri plates (Bibby Sterilin Ltd, Stone, UK).

The plant pathogenic fungus *F. oxysporum* f. sp. *lentis* was originally isolated on selective Komada (1975) medium (KM) from the stems of naturally infested lentil plants. The plants were collected from a diseased experimental plot at the International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria. The pathogenicity of the fungus *F. oxysporum* f. sp. *lentis* was confirmed using lentil cv. Precoz (ILL4605, ICARDA) under pot culture conditions in the crop protection glasshouse at the Department of Agriculture, the University of Reading, UK. Single spore cultures of *T. hamatum* and *F. oxysporum* were subcultured on potato dextrose agar (PDA; 39 g/l, Oxoid, Basingstoke, UK) in a temperature-controlled growth cabinet (Cooled incubator LMS, Kent, UK) at 25±2°C for 14 days with a 12 h photoperiod regime. Pure cultures where stored in the refrigerator at 4°C. For soil inoculation, antagonist conidial suspensions and plant pathogen inoculum production were prepared using the methods described by Erskine and Bayaa (1996), El-Hassan (2004) and El-Hassan and Gowen (2006). Prior to experimental use, fresh cultures of antagonistic and pathogenic fungi were derived from single stock cultures and subcultured on new PDA plates.

Antagonistic activity of *T. hamatum* on agar plates

On PDA plates, 90 mm diameter, dual cultures were set up by placing 5 mm plugs at equal distances (40 mm between the plugs). These inoculation plugs were collected from the growing margins of both *T. hamatum* and *F. oxysporum*. As the controls, 5 mm plugs of the fungal pathogen were added to similar plates. Ten plates for each treatment were sealed, laid in a completely randomized design (CRD) and incubated in a growth cabinet at 25±2°C for 10 days. The experiment was repeated twice. Two types of activity were initially designed: (i) antibiosis: growth-inhibition determined by reduction of the pathogen's mycelium; (ii) competition for nutrients and site: overgrowth and colonization of *F. oxysporum* by *T. hamatum*. The percentage (%) inhibition of *F. oxysporum* radial growth was estimated at 12 h intervals by measuring the radial growth (mm) of the developing colony toward the antagonist until the plant pathogen colony was completely surrounded by the antagonist *T. hamatum*. The percentage of plant pathogen growth inhibition (GI) was calculated according to the following formula:

$$GI = 100 - [100 \times (R_2/R_1)]$$ (Sid Ahmed *et al.* 1999)

where:
GI – inhibition (mm) of *F. oxysporum* vegetative growth
R_1 – radius of the pathogen colony (mm) in the control plate
R_2 – radius of the pathogen colony (mm) in the dual-cultures plate.

At 24 h time intervals, 2 plugs containing *F. oxysporum* mycelium were placed in 10 ml of SDW plus Tween 20. Then, serial dilutions were made and the numbers of conidial spores were determined by using a Fuchs Rosenthal haemocytometer (Scientific laboratory supplies Ltd., Hawksley, UK). An Olympus BH2 light microscope (Olympus optical Co., Tokyo, Japan) was used.

Antagonistic activity of *T. hamatum* on soil plates

Multipurpose peat compost (Roffy Ltd., Bournemouth, UK) soil was passed through a laboratory sieve (3.35 mm pore size). The soil was washed twice with tap water, dried at room temperature, supplemented with 2% glucose and 10% granular lentil seeds (wt/wt). The mix-

ture was moistened with 10% tap water and autoclaved for 30 min on 3 consecutive days. A sample of 15 g of peat-compost mixture was distributed in plastic plates (90 mm diam.). The sample was 70% moistened and lightly pressed to give a flat surface. Plates were inoculated simultaneously with two 5 mm diam. plugs each, of *T. hamatum* and *F. oxysporum* fungi at opposite sides. The plates were placed in plastic boxes in a randomized order and incubated in a growth cabinet at 25±2°C with high relative humidity for 10 days. Two sets of controls were used, one set comprised *F. oxysporum* culture alone and the second comprised *T. hamatum* alone which was inoculated and grown in the same manner as the paired fungi cultures. The fungal growth of *T. hamatum* and *F. oxysporum* were initially determined by the distance (mm) at which the characteristic green sporulation of *T. hamatum* was detected from the inoculum spot.

Antifungal activity of *T. hamatum* filtrate

Culture filtrate was used to demonstrate the possible presence and role of antifungal metabolites on mycelial growth and dry weight of *F. oxysporum* in an attempt to understanding the antagonistic behaviour of *T. hamatum*. The biocontrol fungus *T. hamatum* was grown in 250 ml flasks containing 100 ml of potato dextrose broth medium (PDB) incubated at 25±2°C on a rotary shaker (Gallenkamp, Leicester, UK) at 150 rpm for 12 days. Fungal mats of *T. hamatum* were harvested by centrifugation (Jouan-CR3i centrifuge, Jouan Ltd., Derby, UK) of the culture broth at 4,100 x g, 20°C for 30 min in 150 ml sterile conical plastic tubes (Falcon®; BD Biosciences, Oxford, UK). In order not to destroy the pellet, the supernatant broth solution was carefully drawn off into a sterilized flask. Then, the supernatant was filtered using a sterile Whatman micro GD/X syringe filter (Whatman International Ltd., New Jersey, USA) with a pore size of 0.22 μm. The resulting filtrate was examined under a microscope or by spreading 0.2 ml on TSM plates to confirm it was a fungal-free filtrate. Two types of filtrate-media were prepared: (i) 400 ml filtrates were supplemented with 2% dextrose broth and 2% agar (wt/vol) to make a *T. hamatum* dextrose agar (ThFDA) medium and (ii) 400 ml filtrates were supplemented with 2% dextrose broth (wt/vol) to make the *T. hamatum* dextrose broth (ThFDB) medium. The filtrate media were autoclaved at 121°C for 10 min. Ten ThFDA plates were centrally seeded with a 5 mm diam. plug of *F. oxysporum*. Also, 10 PDA plates seeded with a plug from the fungal pathogen served as the control. The plates were then sealed and incubated in a growth cabinet at 25±2°C. After 10 days, the growth inhibition was analyzed by measuring the radial growth of the *F. oxysporum* colony. The percentage of the mycelia growth inhibition was then computed according to the following formula:

$$GI = 100 - [100 \times (R_2/R_1)]$$

where:
GI – inhibition (mm) of vegetative growth of *F. oxysporum*
R_1 – radius of the pathogen colony (mm) in the control plate
R_2 – radius of the pathogen colony (mm) in the ThFDA plate.

The 75-ml ThFDB broth flasks were inoculated with 2 plugs (5 mm diam.) taken from a *F. oxysporum* culture and incubated on a rotary shaker (150 rpm) at 25±2°C for 10 days. The same volume of sterile DB (dextrose broth) medium was inoculated and used for the control. After incubation, the growth of *F. oxysporum* in the filtrate was harvested by centrifugation at 4,100 x g, 25°C for 30 min. Next, the mycelia was dried in the oven (Memmert, UK) at 45°C for 6 hours and the weight was determined.

Cellulolytic activity of *T. hamatum*

The cellulolytic activity of *T. hamatum* was tested to evaluate the production of cellulolytic enzymes by hydrolyzing the carboxymethylcellulose (CMC). The CMC utilization by *T. hamatum* was measured by the growth rate of *T. hamatum* on this medium and clear zones detected by the Congo red method (Sazci *et al.* 1986). Salt solution agar plates containing 1% CMC were prepared from the following (g/l): 1.4 $(NH_4)_2SO_4$, 0.3 $NH_2CO.NH_2$, 2.0 KH_2PO_4, 0.3 $CaCl_2$, 0.3 $MgCl_2.6H_2O$, 0.005 $FeSO_4.7H_2O$, 0.016 $MnCl_2.H_2O$, 0.014 $ZnCl_2.H_2O$, 0.002 $CoCl_2.6H_2O$, and 10.0 CMC (Sigma Aldrich, Montana, USA) and pH 5.6. A sterile cork borer was used to make the 3-mm in diameter wells in the centre of the CMC plates. The wells were carefully filled with 0.2 ml aliquots of conidia spores (10^8 spore/ml) of *T. hamatum*. After 5 days of incubation, the plates were examined for zones of CMC hydrolysis enzyme activity. A clear halo around the colony of *T. hamatum* showed that there was hydrolysis activity. The hydrolysis zones were visualized by flooding the cultures with an aqueous solution (0.1%) of Congo red (Sigma) and shaking at 50 rpm for 15 min. The Congo red solution was then poured off and plates were further flooded with 1 M NaCl solution. The fungal growth was stopped by flooding the CMC plates with 1 M HCl (pH 0.1) which changed the dye to a blue-violet colour. The diameter (mm) of cellulolytic zones was measured in 10 replicates and the experiment was repeated twice.

Mycoparasitic activity of *T. hamatum*

The hyphal interactions between *T. hamatum* and *F. oxysporum* were studied for mycoparasitic ability using a pre-colonized agar plate method as described above. Mycelium contacts, intersections, and subsequent overlap of both the *T. hamatum* and the pathogen hyphae began to form 2–3 days after incubation in the dark at 25°C.

Light microscopy

Mycoparasitic activities were observed microscopically for any morphological changes in the mycelial growth of *T. hamatum* and *F. oxysporum*. At early stages of contact, mycelium agar 10x20 mm strips were removed from the interaction zone, placed on sterilized microscope slides and observed under oil immersion at x100 magnification using the Olympus BH2 microscope. Mycoparasitic manifestations at different stages of development were recorded and microphotographed using an Olympus camera with a 100 Fujichrome positive film and compared with hyphae of the same age in the control plates.

Scanning electron microscopy

Plugs, which were 10 mm in diam., were excised from the interaction zone of 3 to 7 days old dual colonies. Samples were processed for scanning electron microscopy according to the standard preparation protocol described previously by Mycock and Berjak (1991). The simple preparation was carried out by fixing the mycelial samples in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0). After incubation for 6 h at 4°C, the samples were washed 3 times in 0.1 M phosphate buffer for 10 min each and dehydrated for 20 min in a graded ethanol series (30, 50, 60, 70, 80, 90 and 100%). Dehydrated samples were then critical-point dried using liquid carbon dioxide. Double sided sticky tape was used to mount the samples on aluminium stubs. Then, the samples were coated with gold particles in a sputter coater and frozen. The coated specimens were observed with a research grade conventional scanning electron microscope (SEM; LEO Electron Microscope Model 1450VP, Carl Zeiss SMT AG Com., Germany). Electron micrographs were taken at various magnifications in situ by an SEM operating at 20 kV.

Antagonist-pathogen interactions under controlled conditions

Loam soil and silver sand (Roffy Ltd., Bournemouth, UK) were put separately into sterilized polypropylene bags, moisture maintained at 10%, and then autoclaved three times for 30 min at 121°C. After autoclaving, the loam soil and sand were air-dried on plastic trays at room temperature for 1 day and mixed in a ratio of 2:1 (loam/sand; vol/vol). Plastic pots (9x9x7.5 cm) were plugged from the bottom with a Whatman filter paper and each filled with 250 g soil mixture. Seeds of lentil (cv. Precoz ILL4605) were surface-sterilized by agitating in 5% (vol/vol) household bleach for 10 min, washed 5 times in SDW, and dried for 2 h. One seed was sown in each pot. Seedlings, which were two weeks old, were inoculated with a 6 ml suspension ($2.5x10^6$ spores/ml) of *F. oxysporum* f. sp. *lentis* into 2 holes (1 cm diam., 3 cm depth) on both sides of the seedlings. At the same time, either 5-ml of *T. hamatum* suspension with a final concentration of $5x10^8$ conidia/ml or 10 ml of *T. hamatum* filtrate were individually dripped in 2 new holes (3 cm deep) around the seedlings in the respective treatments. Sterile distilled water was used on un-inoculated control seedlings. Afterwards, holes were promptly covered to prevent drying and care was taken to avoid over watering in the early days of inoculation. The experiment was set up in a completely randomized design with 3 replications (5 pots in each replication) under controlled conditions in a growth chamber at 25°C. There was a 14 h photoperiod provided by cool-white fluorescent light and 10 h of dark. Plants were treated every 10 days with a slow release fertilizer at 0.5 g/l of water (Soluble feed plant-NPK-12-10-12, Homebase Ltd., Reading, UK).

Biocontrol activity was determined by the severity of symptoms produced by the plant pathogen. The disease severity of pathogen infection on individual plants was assessed at 5 days intervals once there was an appearance of > 30% (DSI ≥ 3) of disease symptoms on the control plant, A rating scale of 1 to 9 (Bayaa and Erskine 1990) was used: 1 = no symptoms; 3 = yellowing of the basal leaves only; 5 = yellowing on 50% of the foliage; 7 = complete yellowing of the foliage, flaccidity of the top leaves, partial drying, and 9 = the whole plant or a unilateral shoot is wilted and/or dry. The disease index of individual plants were transformed to percent (%) disease values from numerical ratings by using the conversion formula:

$$DSI = [\sum (\text{number of plants in the rating} \times \text{rating numbers})/(\text{total number of plants investogated} \times \text{maximum disease rating})] \times 100$$

where:
DSI – Disease severity index
$\sum$ – number of plants in the rating x rating numbers/total number of plants investigated.

Antagonist-pathogen interactions in the glasshouse

Plastic seed trays (22x18x5 cm) were filled up with 2 kg of loam/sand (2:1 vol/vol) soil mixture. A template was used to make 2 furrows (2 cm deep and 20 cm long), and ten lentil surface-sterilized seeds of 'Precoz' ILL4605 (a highly susceptible variety) were evenly sown in each tray. The trays were placed on a glasshouse bench where the temperature was 25±5°C day/night. Two weeks after planting, each tray was inoculated with a 60 ml spore suspension of *F. oxysporum* (at the above concentration) into 2 cm deep furrows, uniformly on both sides of the seedlings. The pathogen inoculum was applied after this period to avoid developing wilt symptoms at the seedling stage. At the time of the pathogen inoculation, either 40-ml of *T. hamatum* spore suspension with a final concentration of $5x10^8$ conidia/ml or 50-ml of *T. hamatum* filtrate were distributed equally around the seedlings in the respective treatments. Plants were carefully watered by hand every 3–4 days, and fertilized with soluble feed plant-NPK-12-10-12 at 10 days intervals after the antagonist-pathogen inoculation. At the flowering stage, plants were exposed to water stress to enhance the development of *F. oxysporum* in the vascular system and produce the symptoms of wilt. The experiment was set up in a completely randomized design with 3 replications (2 trays each replication, each tray had a sample size of 10 plants) and experiment repeated twice.

The treatments employed in both experiments were: (i) pathogen-inoculated seedlings (*F. oxysporum* only); (ii) biocontrol-inoculated (*T. hamatum* spore suspension); (iii) biocontrol-filtrate-inoculated (*T. hamatum* filtrate only); (iv) pathogen-biocontrol-inoculated (*F. oxysporum* + *T. hamatum* spore suspension); (v) pathogen-biocontrol-filtrate-inoculated (*F. oxysporum* + *T. hamatum* filtrate) and (vi) Un-inoculated seedlings (drenched with tap water). The use of the *T. hamatum* treatment was to test whether or not the antagonistic isolate can induce any like-disease symptoms or abnormalities in the plants. Biocontrol activity was measured by the incidence of wilt produced by the pathogen on treated plants at time intervals, upon appearance of > 30% disease symptoms on the control treatment. Percent of wilt incidence was recorded and calculated by dividing the number of infested plants by the total number of plants remaining healthy in each tray and multi-

plying by one hundred. An incubation period for disease development was established as the number of days taken for the disease index (DI > 0). The control plants and those treated with *T. hamatum* and *T. hamatum* filtrate grown in un-inoculated, pathogen-free, soil were not included in the statistical analysis of disease incidence.

Population densities, the colony forming units (cfu/g soil) of the plant pathogen and the biocontrol fungus were individually quantified at 10 and 14-day intervals after inoculation in growth room and glasshouse, respectively. Five grams of rhizosphere soil were weighed and dried in plastic dishes in the laminar-airflow cabinet. Three 1-g sub-samples of sieved soil were placed individually in screw-cap glass jars containing 99 ml of SDW plus Tween 20. Serial dilutions were made from the soil washings, vortexed for 30 s, and only 0.2 ml aliquots from 5-fold dilutions were plated onto each of the TSM and KM agar plates. Five plates were used from the final dilution and incubated at 25±2°C for 5 days. After incubation, cfus of the biocontrol and plant pathogen were visually counted and expressed as cfu per gram of air-dried soil.

At harvesting time, asymptomatic plants were collected randomly. The development of the pathogen and the biocontrol fungus in the vascular system was determined by plating 10 surface sterilized segments of plant stems (stem divided to 10 mm long segments) on each KM and TSM plates using the method described by El-Hassan and Gowen (2006). After 10 days, the number of segments which produced *F. oxysporum* colonies (examined under a microscope for sporulation) of each plant/plate were counted. The competitive colonization percentage (%) was calculated as follows:

CI = [number of stem segments colonized by *F. oxysporum*/total number of stem segments] x 100

where:
CI – Colonization index.

The biocontrol efficiency of endophytic *T. hamatum* was presented as a percent reduction in colonized vascular tissues by the pathogen *F. oxysporum*.

Statistical analysis

The percentage values of pathogen growth inhibition, wilt incidence and *Fusarium* plant colonization were transformed to their square root values (SQRTx+0.05) to normalize the variance. Rhizosphere populations and plate spore production of *T. hamatum* and *F. oxysporum* were also transformed to logarithmic base (Log_{10} x+1) of cfu values to normalize the data. Data were analysed according to standard analysis of variance (ANOVA) procedures by the GenStat 11th edition package (Lawes Agricultural Trust, Rothamsted Research, Harpenden, UK) to determine which bio-control treatment produced a higher mean of growth inhibition, a higher mean of rhizosphere population, a lower mean of wilt and disease incidence, and a lower of pathogen development in stem tissues than the control. If a significant *F*-test was obtained among the treatments, significance of difference among means was performed using Fisher's protected least significant difference (LSD) and Duncan's multiple range test (DMRT) at $p \leq 0.05$.

RESULTS

Antifungal activity

On agar plates, *T. hamatum* grew fast, colonized the whole plate and stopped the radial growth of the pathogen at an average of 20 mm diameter (Fig. 1A). The reduction of mycelial growth and spore production of the pathogen was significantly ($p \leq 0.05$) higher in the dual culture compared with the pathogen control due to the competition for available nutrients and space (Fig. 1A, D). The first noticeable contact between hyphae of *T. hamatum* and *F. oxysporum* happened within 56 h post-inoculation. In the following hours, the mycelium of *T. hamatum* rapidly overgrew, completely surrounded, and aggressively colonized the hyphae of the pathogen. Then the mycelium sporulated abundantly by forming hemispherical conidial pustules of greenish ellipsoidal conidia spores (Fig. 1A, B). The level of inhibition was particularly well developed with the increase in the age of the fungal cultures, when the pathogen had little space to grow, and when there was no clear zone of inhibition between the antagonist and pathogen in any of the 10 plates (Fig. 1A, B). During the 72 hours of incubation, the percent inhibition in the mycelial growth of *F. oxysporum* was significantly ($p \leq 0.05$) increased up to 84.26% (9.18 SQRT) in dual culture plates compared to 0.29% (0.44 SQRT) in the pathogen control (Fig. 1B). At this time, percent colonization of the co-culture plate by *T. hamatum* reached 100%. The cfus of *F. oxysporum* had significantly ($p \leq 0.05$) decreased to 3.98 Log_{10} ($9.8x10^3$) cfu/ml in dual cultures compared to 5.66 Log_{10} ($4.6x10^5$) cfu/ml in the pathogen control, in 240 hours post-inoculation (Fig. 1C). Healthy and extensive hyphal growth with abundant sporulation of *F. oxysporum* was evident on the control plates (Fig. 1A, B, C).

On soil plates, *T. hamatum* produced a massive growth of spores on soil and completely colonized the soil mix inoculated with the pathogen during the 10 days of incubation. The antagonistic activity of *T. hamatum* was more intensive and dense on the surface of soil plates than on agar plates in the presence of *F. oxysporum* (Fig. 1A, D). After 10 days of incubation, there was no apparent growth of *F. oxysporum* in the presence of *T. hamatum* compared with *F. oxysporum* alone (Fig. 1D). After 120 hours post-inoculation, the percent inhibition in the mycelial growth of *F. oxysporum* significantly ($p \leq 0.05$) increased up to 67% (8.21 SQRT) compared to 0.05% (0.22 SQRT) in the pathogen control after 120 hours post-inoculation (Fig. 1E). The cfus of *F. oxysporum* had significantly ($p \leq 0.05$) decreased to 4.7 Log_{10} ($1.2x10^4$) cfu/ml in dual cultures compared to 5.93 Log_{10} ($8.5x10^5$) cfu/ml in the pathogen control at the same time of incubation (Fig. 1F).

On both media of *T. hamatum* filtrates, the inhibition in the hyphal growth, dry weight, and spore production and germination of the pathogen was significantly higher compared with the control displaying the strongest fungicidal activities of the secondary metabolites in the filtrates (Fig. 2). The percent inhibition in the mycelial

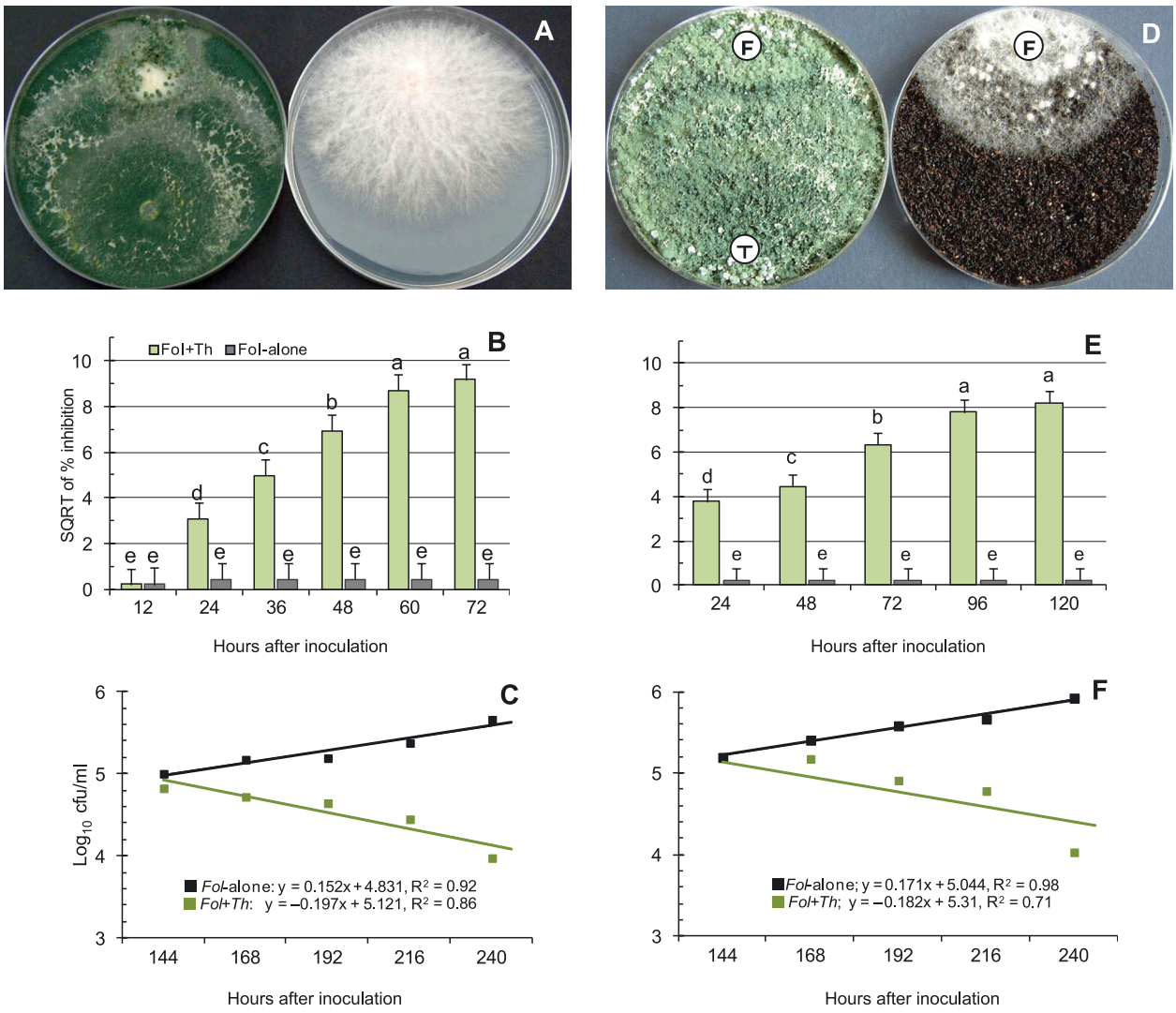

Fig. 1. *In-vitro* growth and spore inhibition of *F. oxysporum* f. sp. *lentis* by *T. hamatum* on agar and peat-substrate plates after 10 days; (■) *F. oxysporum* + *T. hamatum* (left plate) and (■) *F. oxysporum* alone (right plate). (A–C) Inhibitory activity of *T. hamatum* on hyphal growth and spore production of *F. oxysporum* on PDA; (D–F) Inhibitory activity of *T. hamatum* on hyphal growth and spore production of *F. oxysporum* on soil plates. Data are means of 5 replications. Vertical error bars represent standard errors of differences of means. Means topped by the same letter are not significantly different from each other according to Duncan's comparison test ($p \leq 0.05$)

growth of *F. oxysporum* and dry weight was 100% compared to the pathogen control after 7 days of incubation (Fig. 2B, D). When 0.2 ml aliquots of broth filtrate from shaken cultures was removed and placed on KM plates, the germination of the spores was reduced significantly and the germ tubes were unable to develop and grow normally (data not show). The conidia spores of *F. oxysporum* failed to germinate and the mycelia failed to grow after 7 days of incubation in *T. hamatum* culture filtrate (Fig. 2A, B). This failure was an indication that the antibiotic compounds produced by the antagonist *T. hamatum* are not only fungistatic but also fungicidal. The thermostability of antifungal compounds by autoclaving at 121°C for 10 min did not affect the fungicidal activity of the filtrate against *F. oxysporum* compared to the control (Fig. 2). Additional microscopic observations clearly illustrated the lytic effect of *T. hamatum*-filtrate on pathogen hyphae after 36 hours post-inoculation (data not show).

Cellulolytic activity

The CMC medium is found to be a suitable carbon source for cellulytic enzyme production. From the first day of incubation, the filamentous fungus *T. hamatum* exhibited high cellulytic activity. The mycelial growth increased and the zones of hydrolysis of cellulosic sources were produced which reached a 39–40 mm diameter on CMC agar plates within 5 days of incubation (data not show). The highest cellulolytic activity in the width of a 3.4 mm clear zone was detected on CMC plates when the plates were stained with Congo red and fixed with 1 M HCl (data not show). The cellulytic activity of *T. hamatum* suggests that throughout the hydrolysis of cellulosic sources, as the incubation time is increased, the viscosity of CMC medium is continuously decreased. The activities of the cellulase enzymes may show improvement when compared to that at the beginning of hydrolysis.

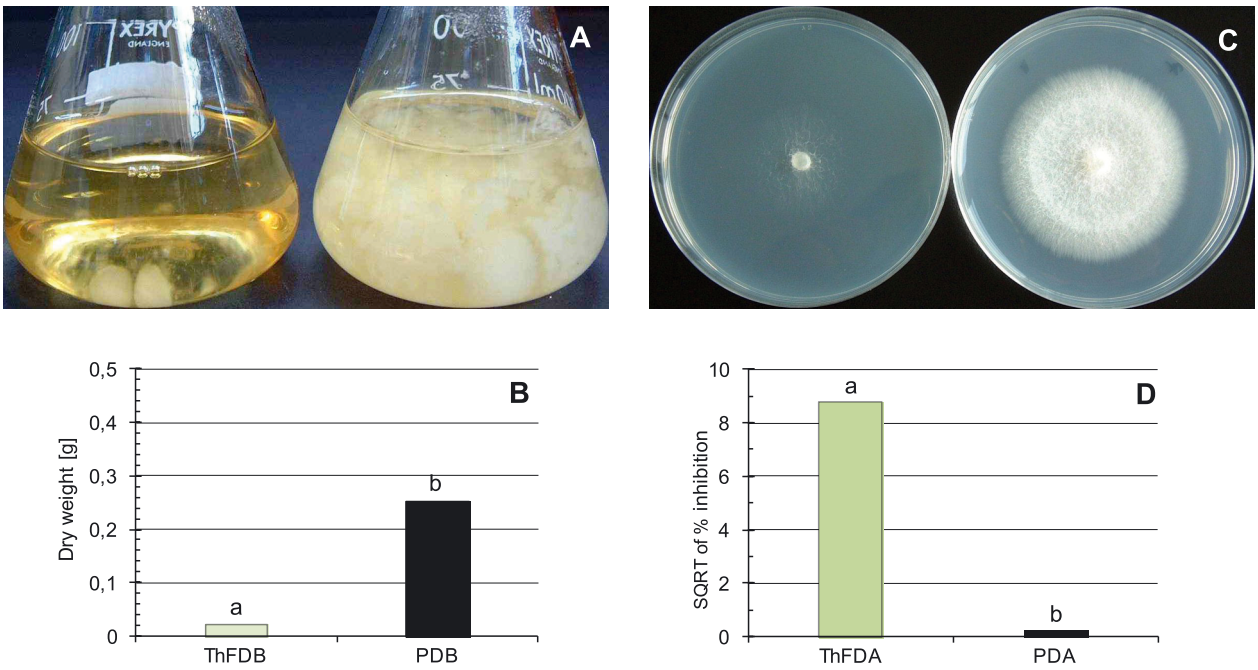

Fig. 2. Antifungal activity of *T. hamatum* culture-filtrates on *F. oxysporum* f. sp. *lentis* growth on broth cultures and agar plates after 7 days of incubation. (A–B) Inhibitory activity of *T. hamatum* filtrate broth (ThFDB, left flask) on hyphal growth (dry weight) of *F. oxysporum*, (C–D) Inhibitory activity of *T. hamatum* filtrate agar (ThFDA, left plate) on hyphal growth of *F. oxysporum* on PDA. Data are means of 5 replications. Means topped by the same letter are not significantly different from each other according to Duncan's comparison test ($p \leq 0.05$)

Mycoparasitic activity

Scanning light and electron microscopical studies of the hyphal interactions showed that *T. hamatum* was a successful and active mycoparasite of *F. oxysporum*. The first apparent physical contact between hyphae of *T. hamatum* and *F. oxysporum* occurred within 56 hours after inoculation on PDA plates. In the following days, various parasitic events, physiological developments, and morphological changes were observed as follows: (i) a rapid colonization of the PDA and soil plates by the antagonist in which *T. hamatum* grew abundantly around and over the hyphae of *F. oxysporum*, established physical contact causing inhibition of *F. oxysporum* hyphal growth (Figs 3A, B, 4A); (ii) hyphal overgrowth and mass sporulation of *T. hamatum* on the hyphae of *F. oxysporum* in the contact zone and over the pathogen (Figs. 3, 4A); (iii) in the zone of contact, *T. hamatum* was observed to have attached and developed appressoria-like structures on the hyphae of *F. oxysporum*, causing mycelial vacuolate and cytoplasmic coagulate of the host pathogen (Fig. 3C, arrows); (iv) the hyphae of *T. hamatum* was observed to more frequently develop hyphal branches around the hyphae of *F. oxysporum*, some of which appeared to penetrate the surface hyphae of the pathogen and were further advanced by secreted enzymes through the cell wall (Fig. 3D, arrows); (v) the antagonist, eventually, established aggressive parasitic contact and made morphological changes by coiling densely and tightly around the pathogen hyphae, even at early stages of interaction (Figs. 3E, F, arrows); and (vi) it was observed that when *T. hamatum* proceeded to penetrate the pathogen cell wall, it utilized the cellular contents causing collapse of *F. oxysporum* hyphae due to the loss of turgor pressure, thereby, destroying the cell wall integrity (Figs. 4B, C) in comparison with the hyphae of *F. oxysporum* grown in a single culture (Fig. 4D).

Antagonist-pathogen interactions under controlled conditions

The mean populations of *T. hamatum* ($5x10^8$ cfu) in the co-inoculated treatment had significantly ($p \leq 0.05$) increased up to 9.92 Log_{10} ($9.92x10^9$ cfu) and slightly decreased to 7.69 Log_{10} ($5.74x10^7$ cfu) per gram of air-dried soil between the 10th and 40th days, respectively, after planting. When using only the *T. hamatum* treatment, the population increased up to 8.80 Log_{10} ($6.46x10^8$ cfu) and decreased to 5.94 Log_{10} ($5.54x10^5$ cfu) per gram of soil during the same period as detected on the TSM plates (Fig. 5A). However, the total number of *T. hamatum* had significantly ($p \leq 0.05$) higher colonization percentages in the combined treatment (7.69 Log_{10} cfu/g soil) with the pathogen than when it was alone (5.94 Log_{10} cfu/g soil) in the vicinity of plant roots after 56 days of planting.

The disease severity of lentil increased over time with symptoms first visible 38 days after planting in the growth room. Results have revealed that the soil drench with a spore suspension of *T. hamatum* or its culture filtrate significantly ($p \leq 0.05$) decreased disease severity. Only 40% (6.38 SQRT) and 55% (7.45 SQRT) of plants died, respectively, compared to 95% (9.74 SQRT) of plants killed (in the final score) in the corresponding controls after 58 days. The results clearly show that the reduction in disease severity over time when compared with the control, is probably related to the antifungal activity of *T. hamatum* and its filtrate in the rhizosphere in the 15 days after application (Figs 5A, B). The antagonist and its culture filtrate reduced disease development on the plants co-inoculated with the pathogen. The final disease index values were significantly lower and the incubation period significantly higher than in plants inoculated with the pathogen only (Fig. 5B). There were no symptoms observed in the control plants

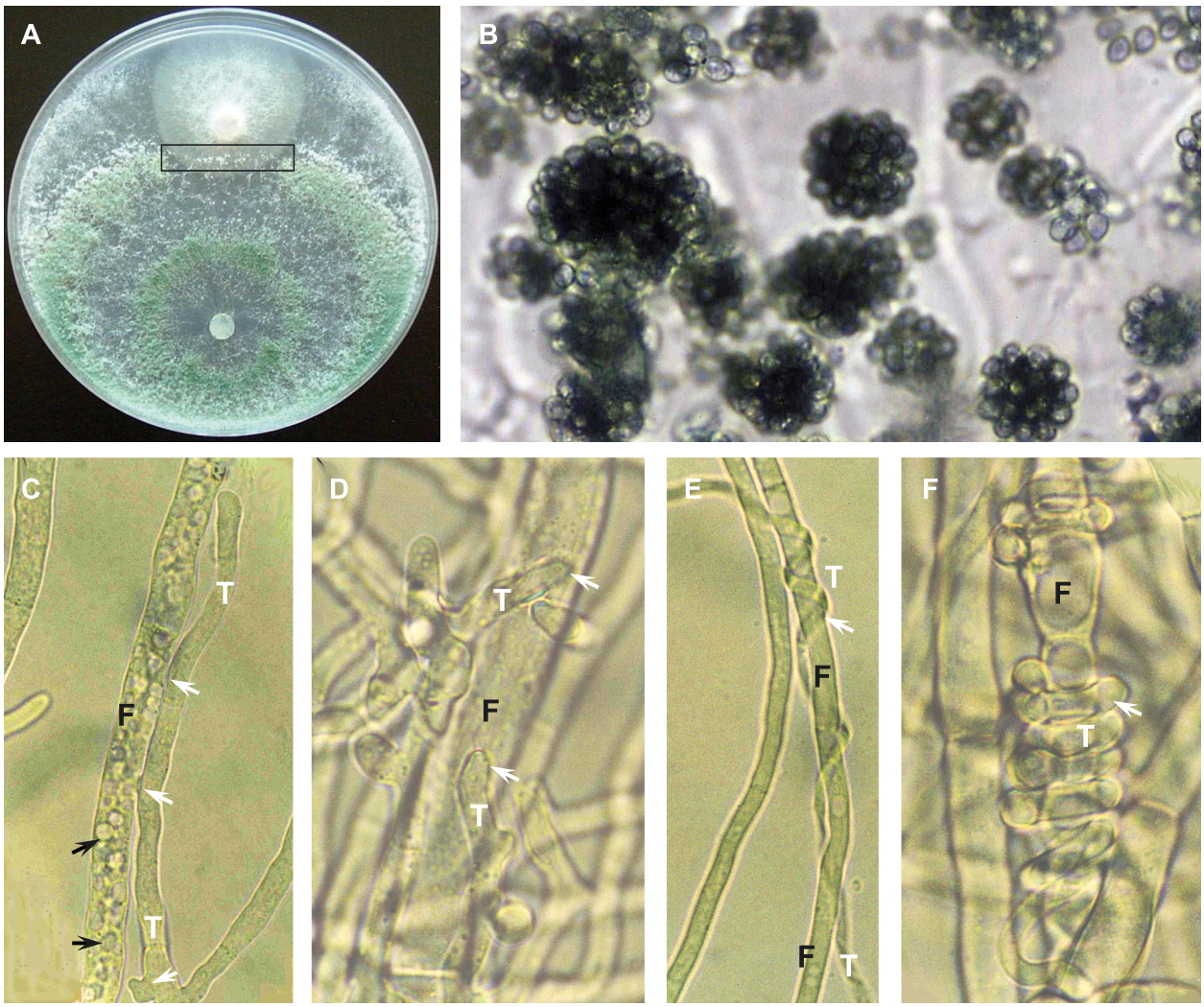

Fig. 3. Light micrographs of *T. hamatum* hyphae in contact with the hyphae of *F. oxysporum* in dual cultures on PDA plates between 3 to 7 days of incubation in the dark. F: *F. oxysporum*, T: *T. hamatum*. (A) Overlap and contact zone between *T. hamatum* and *F. oxysporum* (rectangle showing source of antagonist-pathogen mycelial samples for microscopic studies); (B) *T. hamatum* grew over and colonized the hyphae of *F. oxysporum*, formed hemispherical pustules and produced a huge number of greenish conidial spores by the second day of incubation in the light; (C) Hyphae of *T. hamatum* alongside a vacuolated (black arrows) hyphae of *F. oxysporum* and the developed appressoria (white arrows) to which it has become attached 3 days after inoculation, (D) Hyphal branches of *T. hamatum* attaching (arrows) to the hyphae of *F. oxysporum* 4 days after inoculation; (E) Hyphae of *T. hamatum* winding around (arrow) hyphae of *F. oxysporum* 5 days post inoculation, and (F) Hyphae of *T. hamatum* coiling excessively around the hyphae of *F. oxysporum* 7 days after inoculation (x1,000 Mag)

(*T. hamatum* alone) nor in plants grown in un-inoculated soil (tap water treatment). The pathogen was not isolated from the lentil vascular tissues of those treatments.

The antagonist colonization percentage was developed as a general assessment of the ability of *T. hamatum* to establish an endophytic relationship with lentil plants in an attempt to protect the plants directly from the initial pathogen infection. Isolating *T. hamatum* from vascular tissues indicated the isolate was living inside the plant tissue and is therefore an endophyte of lentil stem tissues (Fig. 5C). The use of *T. hamatum* and its filtrate significantly ($p \leq 0.05$) inhibited the percent infection and development of the pathogen *F. oxysporum* in the vascular tissue of lentil plants to no more than a mean of 27% (4.81 SQRT) and 63% (7.96 SQRT) stem colonization, respectively, compared to100% (10.00 SQRT) in the control (pathogen-inoculated) plant (Fig. 5C).

Antagonist-pathogen interactions in the glasshouse

Assessments of *T. hamatum* population density on TSM at 14 days intervals showed constant and progressive rhizosphere colonization. At all sampling dates, the antagonist population was significantly ($p \leq 0.05$) higher in the combined treatment (antagonist-pathogen) than when *T. hamatum* was applied alone (Fig. 6A). The applied populations ($5x10^8$ cfu) of *T. hamatum* had significantly ($p \leq 0.05$) increased up to 9.94 Log_{10} ($8.7x10^9$ cfu), rapidly decreased to 6.14 Log_{10} ($3.9x10^6$ cfu) and then increased up again to 8.57 Log_{10} ($5.5x10^8$ cfu) g air-dried soil at 14, 42 and 56 days post inoculation, respectively, when combined with the pathogen (Fig. 6A). In the treatment *T. hamatum* alone, the population had increased up to 8.41 Log_{10} ($5.7x10^8$ cfu), decreased to 5.12 Log_{10} ($1.4x10^5$ cfu) and then increased up again to 5.53 Log_{10} ($4.5x10^5$ cfu) g soil at the same sampling dates as determined by TSM (Fig. 6A). During the 56 days of soil inoculation, *T. hamatum* yielded better mean

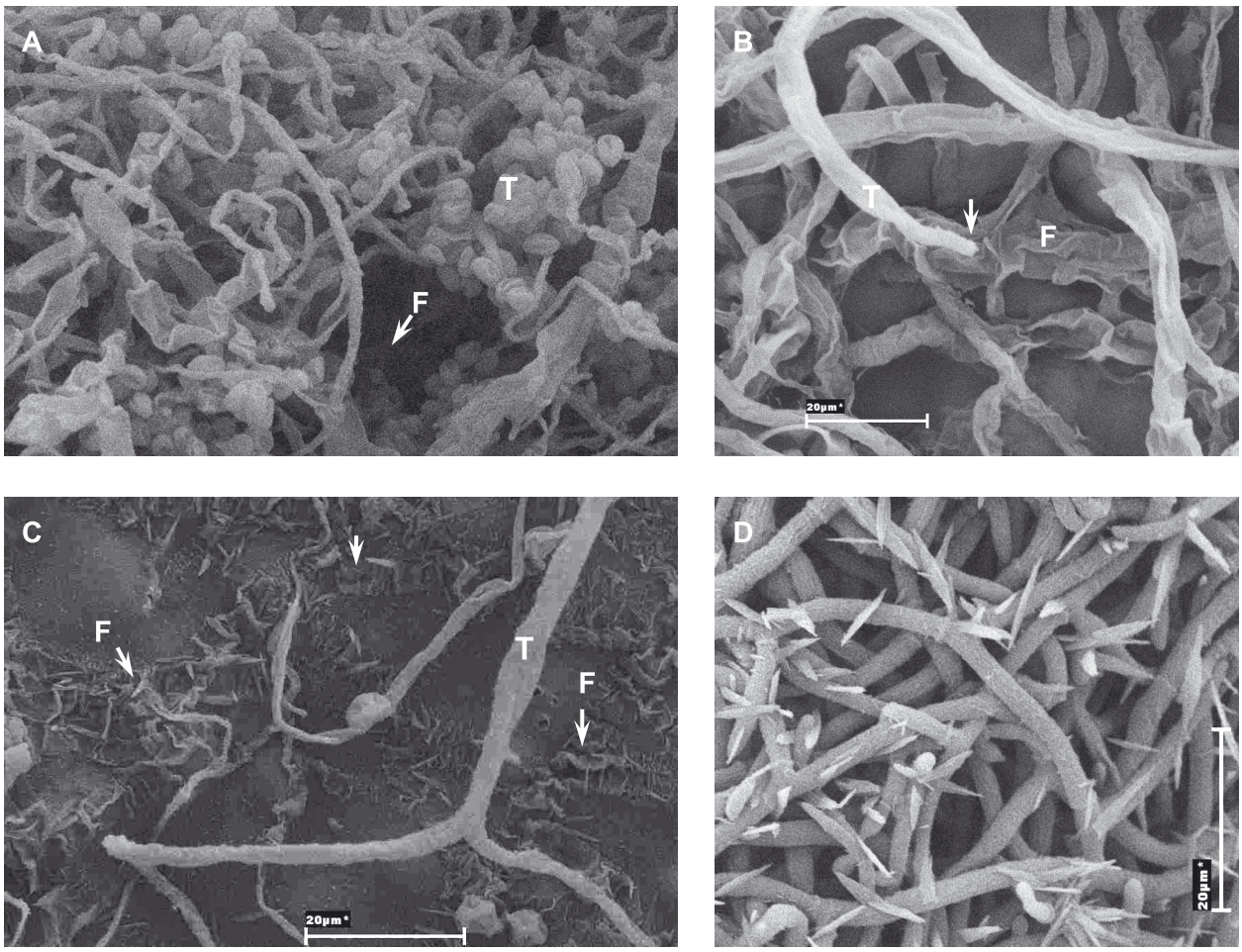

Fig. 4. Electron micrographs on mycoparasitism of the *F. oxysporum* hyphae by the hyphae of *T. hamatum* in dual cultures 7–12 days after inoculation on PDA plates. F: *F. oxysporum*, T: *T. hamatum*. (A) *T. hamatum* biomass growth and spores which adhered onto the hyphae of *F. oxysporum* (x1000 Mag) 7 days after inoculation; (B) *T. hamatum* hyphae tip attached to and penetrating (arrow) the hyphae of *F. oxysporum* (x3000 Mag) 8 days after inoculation; (C) loss of turgor and marked hyphae collapse (arrows) of *F. oxysporum* 12 days after invasion (x3000 Mag), where *T. hamatum* hyphae continue to look normal; and (D) *F. oxysporum* hyphae alone (x3,000 Mag) 12 days after inoculation

populations (8.57 Log_{10} cfu) in the combined treatment than when it was used alone (5.53 Log_{10} cfu) compared with the initial applied (8.30 Log_{10} cfu) populations in the rhizosphere of lentil plants.

In the case of the pathogen population, the overall population density ($2.5x10^6$ cfu) of *F. oxysporum* had increased up to a mean of 7.66 Log_{10} ($4.87x10^7$ cfu) and 7.00 Log_{10} ($1.01x10^7$ cfu) g soil when combined with either *T. hamatum* or its culture filtrate, respectively, compared with the pathogen alone (8.59 Log_{10}, $4.05x10^8$ cfu) 14 days after application. At day 28, the density of the pathogen decreased to 6.22 Log_{10} ($3.07x10^6$ cfu) in the presence of *T. hamatum* and increased to 7.41 Log_{10} ($2.92x10^7$ cfu) in the presence of *T. hamatum* filtrate while it was 9.07 Log_{10} ($1.26x10^9$ cfu) g dried soil (Fig. 6B). Subsequently, the population density values of *F. oxysporum* were significantly ($p \leq 0.05$) lower when combined with the bio-control fungus (4.66 Log_{10} cfu) or its filtrate (5.64 Log_{10} cfu) than its population alone (5.98 Log_{10} cfu) after 56 days of application (Fig. 6B). In the rhizosphere, the biocontrol fungal population production rate was less than 1 Log_{10} unit whereas the reduction rate of plant pathogen populations were more than 1.83 Log_{10} units in the co-inoculation treatment compared with initial applied population of *T. hamatum* and *F. oxysporum* on one single plant.

Wilt incidence results have confirmed that soil drench, in either the conidial suspension of *T. hamatum* or culture filtrate, significantly ($p \leq 0.05$) reduced the vascular wilt disease to 33% (5.76 SQRT) and 40% (6.33 SQRT) wilted plants, respectively, compared to 93% (9.66 SQRT) lentil plants killed in the control during the 65 days growth (Fig. 8A). The reduction in wilt incidence over time compared with the control, is probably related to an increase in the population of *T. hamatum* in the rhizosphere. This increase caused a "walling-off" of the pathogen during the period of 14 and 28 days after inoculation (Figs 6, 8A). No apparent differences in the morphological or physiological state were noticed between untreated plants (tap-water treatment) and the one treated with *T. hamatum* alone.

Fungal colonization of the plants by the pathogen was significantly ($p \leq 0.05$) reduced to no more than 40% (6.04 SQRT) in the conidial suspension of *T. hamatum* or culture filtrate treated plants compared to 100% (10.0 SQRT) in the pathogen control treatment (Fig. 8B). In the treated plants, recovery of inoculated *T. hamatum* from roots and stems was an indication of endophytic and competitive

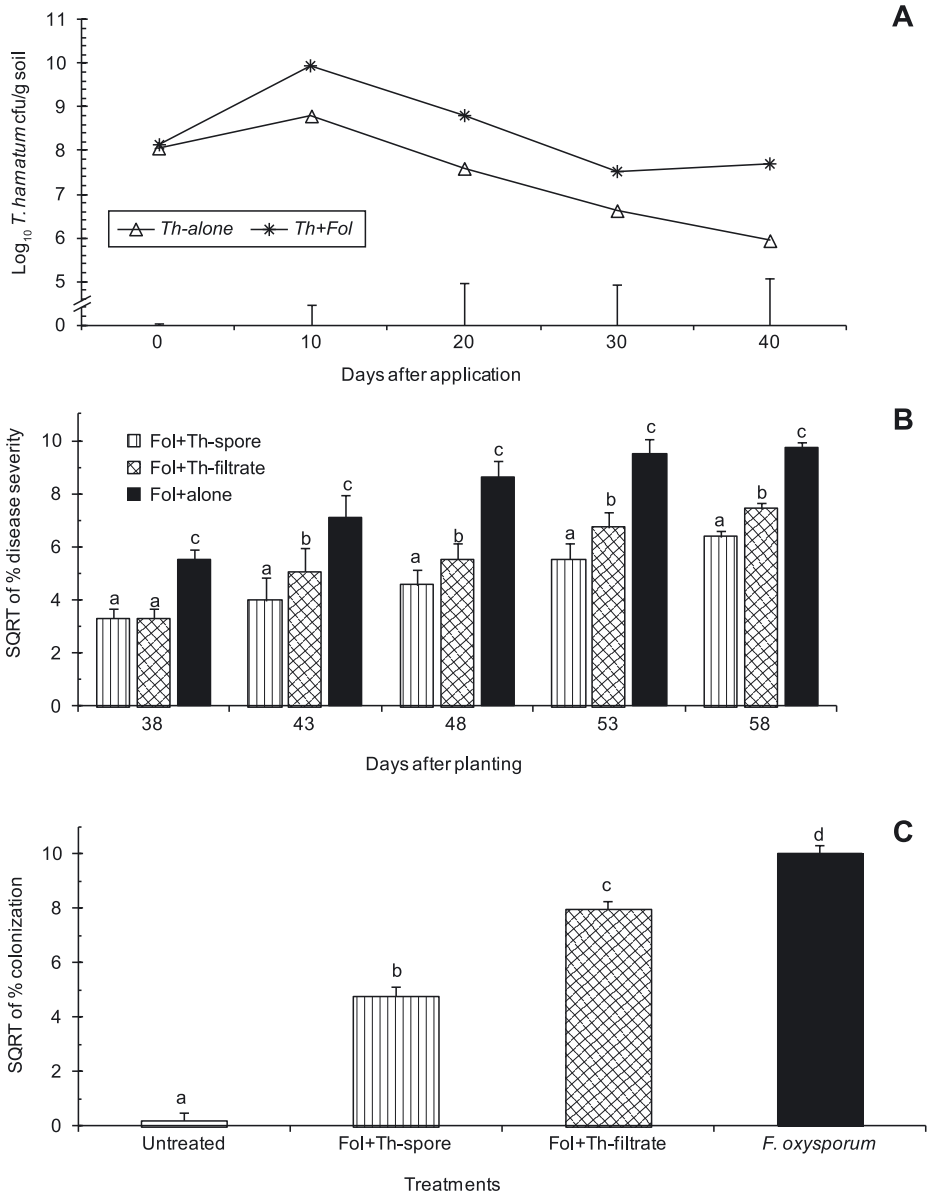

Fig. 5. Effect of conidial suspension of *T. hamatum* and its culture filtrate on disease severity of lentil planted in the growth room: (✳) *T. hamatum* + *F. oxysporum*; (△) *T. hamatum* alone; (□) Untreated (tap-water treatment); (▨) *F. oxysporum* + *T. hamatum* spore suspension; (▩) *F. oxysporum* + *T. hamatum*-filtrate; and (■) *F. oxysporum* alone; treatments were applied to 15-day-old seedlings by drenching the soil with 40 ml ($5x10^8$ conidia/ml) of *T. hamatum*, 50 ml of *T. hamatum* filtrate and 60 ml ($2.5x10^6$ spores/ml) of *F. oxysporum*. (A) Rhizospheric populations (cfu/g soil) of *T. hamatum* were determined by dilution platting on TSM; (B) Disease severity of *F. oxysporum* on individual plants was based on a 1–9 scale: 1 = healthy and 9 = the plant completely wilted and/or dry and expressed as the percentage (%) of diseased plants according to the mentioned conversion formula; and (C) Percent colonization of lentil plants by *F. oxysporum* determined by counting the number of stem-fragments colonized by the pathogen after 10 days of incubation on KM plates. Data are the means of 5 replicated plants. Vertical error bars represent standard error of differences of means. Means topped by the same letter are not significantly different from each other according to Duncan's comparison test ($p \leq 0.05$)

activity. Such indications suggest that successful colonization by the biocontrol fungus can positively influence plant growth and protect the plants from the potential infection and limit the development of *F. oxysporum* (Figs. 7, 8B). In order for *T. hamatum* to colonize the aboveground parts of the plant, the biocontrol fungus would have to stimulate plant growth and decrease the wilt disease to more than 60%, indicating unsuccessful colonization by the pathogen *F. oxysporum* (Figs. 7, 8B, C). However, plant colonization by *T. hamatum* decreased the pathogen infection, increased dry weight and may improve local or systemic resistance in the treated plants (Figs. 7, 8). It is important to note, that colonization of roots and tissues by *T. hamatum* never showed any evidence of abnormalities nor did it induce disease symptoms in its respective treatments (Fig. 7).

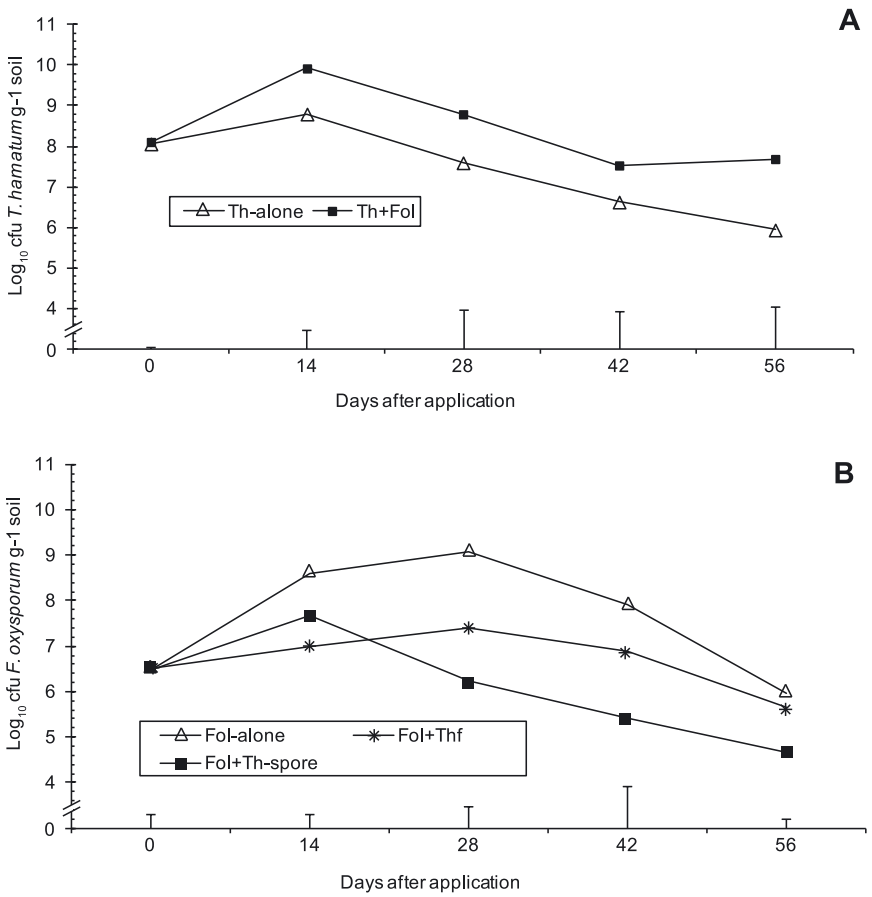

Fig. 6. Effect of soil drench with conidial suspension of *T. hamatum* and its culture filtrate on populations of antagonist-pathogen in the rhizosphere of lentil planted in the glasshouse; (△) *F. oxysporum* alone @ 2.5x10^6 spores/ml; (✳) *F. oxysporum* + filtrate of *T. hamatum* @ 50 ml; and (■) *F. oxysporum* + *T. hamatum* spore suspension @ 5x10^8 conidia/ml. (A) Rhizospheric populations (cfu/g soil) of *T. hamatum* were determined by dilution platting on TSM; and (B) Rhizospheric populations (cfu/g soil) of *F. oxysporum* were determined by dilution platting on KM plates. Data are the means of 5 replicated plant/soil samples. Vertical error bars represent standard error of differences of means

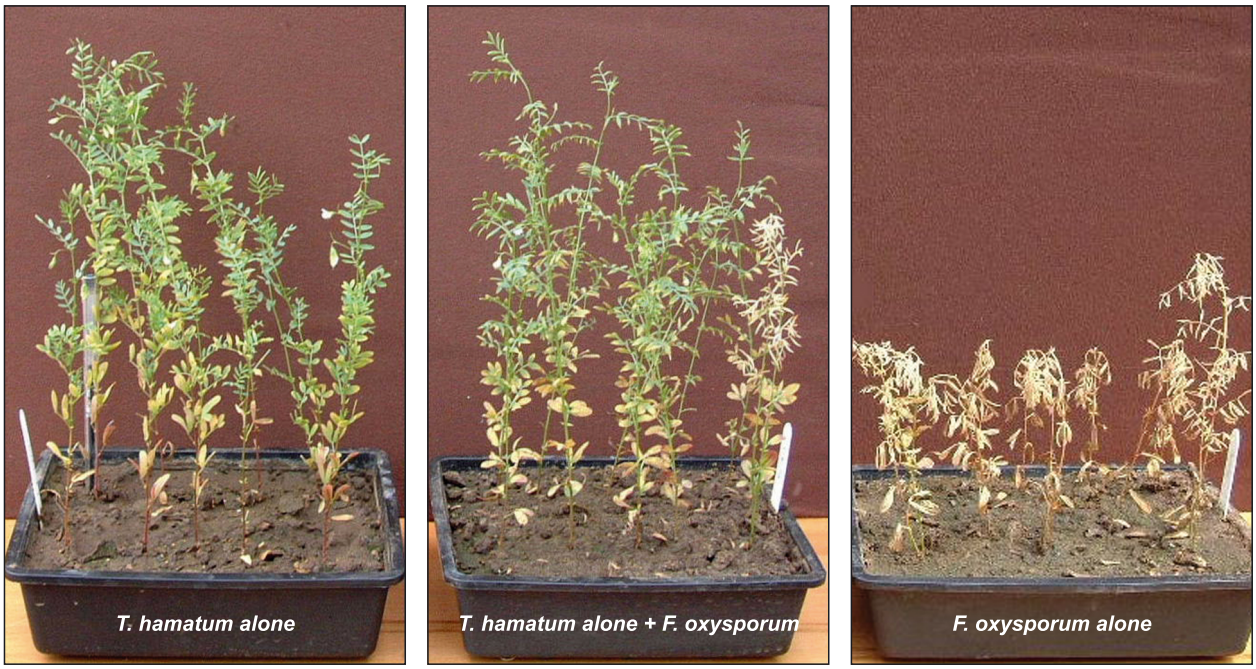

Fig. 7. Effect of conidial suspension of *T. hamatum* on incidence of *Fusarium* wilt of lentil (cv. Precoz) planted in the glasshouse. Conidial suspension was applied to 15-day-old seedlings by drenching natural loam/sand soil with 40 ml (5x10^8 spores/ml) of *T. hamatum* and 60 ml (2.5x10^6 spores/ml) of *F. oxysporum*

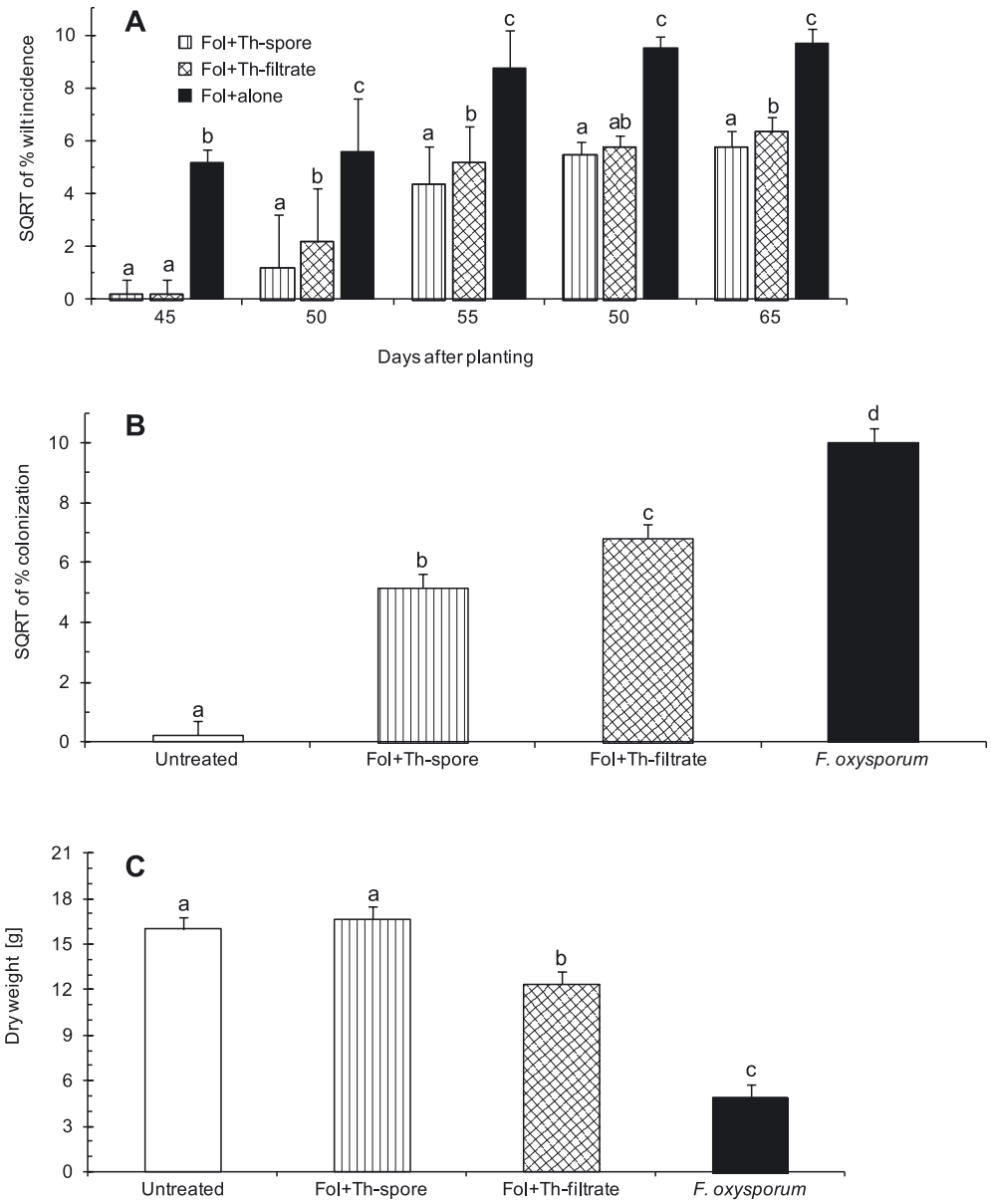

Fig. 8. Effect of conidial suspension of *T. hamatum* and its culture filtrate on incidence of *Fusarium* wilt of lentil planted in the glass-house: (□) Untreated (tap-water treatment); (▨) *F. oxysporum* + *T. hamatum* spore suspension; (▩) *F. oxysporum* + *T. hamatum*-filtrate; and (■) *F. oxysporum* alone; treatments were applied to 15-day-old seedlings by drenching the soil with 40 ml ($5x10^8$ conidia/ml) of *T. hamatum*, 50 ml of *T. hamatum* filtrate and 60 ml ($2.5x10^6$ spores/ml) of *F. oxysporum*. (A) Percentage of wilt incidence caused by the pathogen was significantly different among treatments at each date and (B) Percent colonization of lentil vascular tissue by *F. oxysporum* was determined by *CI* index in counting the number of stem-fragments colonized by the pathogen after 10 days of incubation on KM plates (C) Plant dry weight (g/10-plants). Data are the means of three replicated trays, each with 10 plants. Vertical error bars represent standard error of differences of means. Means topped by the same letter are not significantly different from each other according to Duncan's comparison test ($p \leq 0.05$)

DISCUSSION

The hypothesis was that *T. hamatum* can inhibit the growth of *F. oxysporum* f. sp. *lentis*. The questions to be answered are what are the modes of action involved in this inhibition? Varying modes of hyphal interactions and degrees of inhibition in growth and development of *F. oxysporum* f. sp. *lentis* with *T. hamatum* in co-culture plates, rhizosphere, and endophyte were studied to investigate the mechanisms of control. Understanding the mechanism(s) of action involved in the bio-control processes is of primary importance in establishing these characteristics. Such an understanding can provide much insight about where and when the interaction occurs and how the pathogen will be affected. In order to survive and compete, *Trichoderma* produces a wide variety of toxic and antibiotic metabolites that are active against plant pathogens, such as trichodermin, trichodermol, harzianum A, harzianolide, T39 butenolide, terpenes and polypeptides (Lorito *et al.* 1994; Dickinson *et al.* 1995; Sivasithamparam and Ghisalberti 1998; Vinale *et al.* 2006; Vinale *et al.* 2008; Andrabi *et al.* 2011) and extracellular hydrolytic enzymes (Thrane *et al.* 2000; Eziashi *et al.* 2006) which were involved in the inhibition, competition, and mycoparasitism of phytopathogenic fungi. In this regard, our results support these findings by showing that *T. hamatum* produced a good percentage of antibiosis during the antagonistic and competitive growth which restricted establishment, hyphal growth, and sporulation of the pathogen *F. oxysporum* on agar plates (Figs. 1, 2). The percent inhibition and the ability to grow over pathogen mycelium that occurred on agar plates is considered as antibiosis, parasitism, and competition for nutrients and/or space, as defined by Cook and Baker (1983). The antibiotic metabolites may inhibit the pathogen activities by diffusing toxic chemical substances from the antagonist in the medium or due to its direct effect on the target pathogen by occupying the whole area of growth. On the other hand, the toxicity of antibiotic compounds released in the culture filtrate by *T. hamatum* which completely inhibited the growth of the pathogen mycelium may be similar to the metabolites produced by other *Trichoderma* as mentioned above (Fig. 2). On soil plates, *T. hamatum* is largely active as an antagonist and a saprophyte. It may be considered an important factor for the colonization of the soil and the parasitizing of the pathogen mycelium (Fig. 1). The predominant growth of *T. hamatum* was increased with time of pairing due to the competitive activity between hyphae of *T. hamatum* and *F. oxysporum* for colonizing the available space and utilizing the nutrients in the soil plate. However, the inhibitory effect of *T. hamatum* on hyphal growth, and the development of *F. oxysporum* in soil closely resembled what was observed in the presence of the antagonist on agar plates (Fig. 1).

The fungal cell wall-degrading and cellulolytic enzymes, in addition to antibiotic compounds produced by *T. hamatum,* were thought to be involved simultaneously in the facilitating of parasitism, competition and antibiosis to overcome and maintain *F. oxysporum* inoculum under the pathogenic level (Figs 1–4). Secretion of enzymes and antibiotics by the filamentous fungus on nutrients of carbon sources may be interacted synergistically in the inhibition process of the host pathogen. We suggest that the strong cellulolytic activity of *T. hamatum* provided conclusive evidence that cellulose hydrolysis is one of the mechanisms involved in the antagonistic and parasitic processes. We believe that pathogen hyphal lysis and disintegration could not be reached in the absence of antibiotics and hydrolysis enzymes.

Light and electron microscopical examinations of co-cultures hyphae demonstrated that at early stages of the interactions, the hyphae of mycoparasitic fungus *T. hamatum* established direct contact, multiplied abundantly, coiled aggressively, and attached firmly causing depression of the pathogen hyphal cells (Fig. 3). In the later stages, *F. oxysporum* hyphae showed extreme shrinkage, shrivelling, and cytoplasmic losses when compared with hyphae grown in a single pathogen culture. This comparison supports the hypothesis that mycoparasitism can occur due to nutritional factors provided by the host pathogen (Fig. 4). Therefore, these physiological and morphological changes may justify in part, that production of hydrolytic enzymes by *T. hamatum* such as cellulose, made the parasitism process more efficient and caused degradation of internal hyphae. Subsequently, the structure of the pathogen hyphae collapses (Figs. 4, 5). Our findings parallel a similar order of parasitic events that has been recorded for a number of well-known mycoparasites of *Trichoderma* on *F. oxysporum* and *Verticillium albo-atrum* (Benhamou *et al.* 1999), *F. culmorum* and *F. graminearum* (Pisi *et al.* 2001), *Pythium ultimum* and *Rhizoctonia solani* (Lu *et al.* 2004; Shalini and Kotasthane 2007), *Phytophthora capsici* (Sid Ahmed *et al.* 1999; Schubert *et al.* 2008), *R. solani* (Howell 2003), *Sclerotium cepivorum* (Metcalf and Wilson 2001) and *Thielaviopsis paradoxa* (Sánchez *et al.* 2007).

The biocontrol potential and growth promotion of *Trichoderma* has been widely studied and used. *Trichoderma* saprophytic and endophytic ability to colonize rhizosphere and root material has received quite a bit of attention in the past few years (Metcalf and Wilson 2001; Harman *et al.* 2004; Vinale *et al.* 2006; Hohmann *et al.* 2011). *T. hamatum* has the ability to grow more rapidly on complex carbon, cellulose and nutrient substrates, typical of those found on root surfaces. This ability could be of ecological significance and a characteristic of rhizosphere-competence isolate (Figs. 1, 7). Reisolation from stems and roots confirmed that *T. hamatum* was an efficient endophytic colonizer of the aboveground parts, as well as underground parts. This feature probably played a direct role in the colonization and parasitism mechanisms which might have favoured *T. hamatum's* competitiveness over *F. oxysporum* (Figs. 5–7). Our growth room and glasshouse results indicate that advantageous belowground interactions of the endophytic fungus *T. hamatum* could potentially be translated to the aboveground part. Then there would be reduced disease severity and wilt incidence caused by *F. oxysporum* on lentil plants (Figs. 5–8). This outcome is consistent with previous studies using *T. harzianum*, *T. hamatum*, *T. asperellum* as well as other species of *Trichoderma* in managing diseases and growth promotion on many plant species (Metcalf and Wilson 2001; How-

ell 2003; Yedidia *et al.* 2003; Harman *et al.* 2004; Khan *et al.* 2004; Horst *et al.* 2005; Harman 2006; Vinale *et al.* 2006; Bennett and Whipps 2008; Hohmann *et al.* 2011).

In summary, our results demonstrated that the antagonist *T. hamatum* has varied mechanisms of action; high growth rate, broad spectrum antibiosis, good colonization and rhizosphere competition percentage combined with mycoparasitism. These minimize the disease incidence caused by *F. oxysporum* f. sp. *lentis* through reduction in the pathogen inoculum available to make infection and kill the plants. We suggest that cellulose exploitation by *T. hamatum* plays a role in the rhizosphere competition and parasitism mechanisms, and that is a characteristic criterion for the selection of a novel biocontrol agent. Specifically, in a provided susceptible variety and favoured growth conditions of *F. oxysporum* f. sp. *lentis*, the pathogen was unable to develop infection and produce the disease damage on lentil plants due to the antifungal activities of hydrolytic compounds and the dominance of *T. hamatum* in the vicinity of the roots and plant tissues. Thus, this system of biological control evaluation of the efficacy and mechanisms of interaction would be an appropriate means to characterize and further use *T. hamatum* in the management programme of *Fusarium* vascular wilt disease, where the welfare of farmers depends on the success of lentil cultivation.

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