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In vitro, Antifungal Efficacy of Some *Trichoderma* spp. Against *Fusarium oxysporum* f. sp *betae* Causing Wilt Disease of Sugar Beet Plant

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KEY WORDS

Antifungal activities, *Trichoderma* spp. Optimization, *Fusarium oxysporum* f.sp *betae*, GC/MS.

ABSTRACT

The present study aimed to investigate the antifungal bio-efficacy of four *Trichoderma* spp. (*T. harzianum, T. hamatum, T. viridie* and *T. galaucum*) against *Fusarium oxysporum* f. sp. *betae* (*F. o. b.)* which causing wilt disease of sugar beet. Screening the antifungal activities using dual and disc methods, showed that all the tested *Trichoderma* spp. recorded significant antagonistic effect against *F.o.b.* Out of the four *Trichoderma* spp. *T. harzianum* and *T. hamatum* recorded the highest inhibitory effect on the mycelial growth of *F.o.b*. using dual and disc method where the inhibition reached to 72.53 and 71.30 % and 83.50, 80.00 %respectively. After optimization of cultural conditions of both the selected *Trichoderma* spp., the inhibitory effect on *F. o. b*. were maximized to 85.50 and 82.20% respectively and more inhibitory effect reached to 86.70% was obtained with using the combination between the filtrates of both *Trichoderma* spp. The identification of *F. oxysporum* f.sp *betae, T. harzianum* and *T. hamatum* were confirmed by molecular identification based on internal transcribed spacer (ITS) sequences of rDNA genes. The results showed that *F. oxysporum* f.sp *betae* exhibited similarity 99% to *F. oxysporum* f.sp. *betae,* with acc. no. PP410242, *T. harzianum* exhibited similarity (%) to *T. harzianum* with acc. no. PP410286 and *T. hamatum* exhibited similarity 100% to *T. asperellum* with acc. no. PP410284. Extracts of *T. harzianum* and *T. hamatum* were analyzed using GC-MS to determine the active chemical constituents. The data showed a numerous compounds produced by the two *Trichoderma* spp. possessing high antimycotic property.

Introduction

Among the many devastating soil-borne fungal diseases, *Fusarium oxysporum* is responsible for causing sugar beet wilt disease. There are various formae speciales, and each one is bound to a certain host **(Webb *et al.*, 2012).** Mycelium and chlamydospores of *Fusarium oxysporum* f.sp. *betae* can live in soil and plant debris for a long time. In the proper environmental conditions, a pathogen can invade sugar beet roots and reach the plant's vascular system, where the fungus functions as a "plug" preventing the flow of water through the sugar beet's vascular tissue and causing wilting in the process. *Fusarium oxysporum* f.sp. *betae* causes wilt symptoms in leaves, including yellowing in between the major veins, wilting, chlorosis, and necrosis **(Hill *et al.*, 2010)**. *Fusarium* wilt pathogen-infected plants must be destroyed right away because there is no treatment for them **(Summerell *et al.*, 2011).** *Trichoderma* is a multiactivities fungus that has uses in both industry and agriculture. **Hu *et al.*, (2020)** found that *Trichoderma* spp. is a commonly utilized biological agent for controlling many plant diseases in agriculture. More than 60% of biological pesticides that have been approved worldwide contain it. Science and industry are more interested in this beneficial fungus in agriculture than any other **(Lorito and Woo, 2015)**. *Trichoderma* species have the ability to produce industrially important metabolites, alleviate abiotic stressors, control pests and diseases, and biodegrade xenobiotic substances. One of the more environmentally friendly crop cultivation methods is the use of biocontrol agents, which reduce chemical pollution while simultaneously increasing yield and protecting against diseases **(Hyder *et al.*, 2017).** For biological controls to control plant pathogens, new biocontrol agent products must be developed. The development of these medications necessitates extensive antagonist screening on a broad scale, precise mass production techniques to ensure optimal product quality and quantity, and formulation that maximizes bioactivity, ease of distribution, and preservation **(Jan *et al.*, 2011).** Commercially available biological control formulations of *Trichoderma* spp. include the fungus's asexual reproductive structures, the bulk-generated conidia which produced under *in vitro* conditions, it is important to manipulate nutrients and substrates to boost condition and generate the best development circumstances for many species of *Trichoderma*. These results

suggest that the most significant environmental factors influencing condition in *Trichoderma* are the carbon to nitrogen ratio and pH **(Gao *et al*., 2007)**. *Trichoderma* strains are important because they should be more stress tolerant than the plant diseases against which they will be utilized for biological control **(Kredics *et al*., 2004)**. The morphological and physiological properties of *Trichoderma* spp. are external variables as they do in all microorganisms. pH is the most critical environmental parameter influencing *Trichoderma* strains' mycoparasitic activity **(Kredics *et al*., 2004)**. Investigate the optimal development conditions for these biocontrol agents to increase the antifungal materials and control infections, requires a certain pH value and temperature. At different pH levels between 2 and 7, the isolates of *Trichoderma* showed optimal growth and sporulation rate **(Begoude *et al*., 2007)**. Soil temperatures are critical for the survival of *Trichoderma* species **(Singh *et al*., 2014)**.

Numerous novel and intriguing bioactive metabolites of *Trichoderma* spp., including antioxidants, antibiotics and antivirals have been identified and isolated from soil fungi that are influenced by chemical and physical factors like sources of amino acids, sources of carbon and nitrogen temperature, pH, and incubation duration. These metabolites have implications for the industrial, pharmaceutical, and agricultural sectors **(Strobel and Daisy, 2003)**. The majority of the research found that several ecological and cultural factors influence the formation of secondary fungal metabolites **(Bhattacharyya and Jha, 2011).** Antibacterial activity of *Trichoderma harzianum* was investigated against *Escherichia coli* and *Staphylococcus aureus*. The effects of different nitrogen and carbon sources, temperatures, pH, incubation times and NaCl on antibacterial metabolite synthesis were investigated. *T. harzianum* bioactive metabolite synthesis showed extensive antibacterial efficacy *in vitro* against two strains of bacteria. Glucose and dextrose were discovered to be the finest carbon sources and NaCl the greatest nitrogen sources for the optimal synthesis of bioactive metabolites. The highest levels of bioactive metabolite formation occur at 25°C and pH 7; NaCl has a beneficial effect on bioactive metabolites **(Hateet *et al*., 2021).** *Trichoderma* secretes several enzymes that aid in mycoparasitism, including chitinase and protease. *Trichoderma* also produces antibiotics such as trichodermin and alamethicin, which drive morphological

and physiological changes that lead to hyphae penetration **(Dotson *et al*., 2018)**. Competition for food and space in the rhizosphere is an active kind of antagonism **(Nakkeeran *et al*., 2018).** For instance, *Trichoderma* spp. initiates siderophore release, which chelates Fe^{2+} ions and forms a complex with iron that biocontrol agents can only detect through their membrane-bound protein receptors. This prevents pathogens from obtaining iron **(Vinale *et al*., 2013)**. Phytoalexin synthesis and phenylpropanoid metabolism are two metabolic mechanisms that speed up *Trichoderma* hypersensitivity **(Tripathi *et al.*, 2021).**

A new *Trichoderma* strain has been discovered by analyzing the ribosomal DNA Internal Transcribed Spacer (ITS) region (ITS1—5.8S rDNA—ITS2) and pieces of genes that encode tef-1, endochitinase, RNA polymerase II subunit (rpb2), and calmodulin **(Ribeiro *et al.*,2023).** So, this study was carried out to evaluate the bio-efficacy of *Trichoderma* spp. as an antifungal agent against the sugar beet plant wilt disease-causing *Fusarium oxysporum* f.sp. *betae*.

Materials and methods

The tested *Trichoderma* spp.

Four different pure identified cultures of the bio-agents *Trichoderma* spp. namely *T. harzianum, T. hamatum, T. galaucum* and *T. viridie* were kindly provided by agriculture research center (ARC), plant pathology institute, Giza, Egypt. Each culture of *Trichoderma* spp. was maintained by inoculation separately in Czapek´s dox agar plates and slants and incubated at 25°C for 7 days. The plates and slants containing the different *Trichoderma* spp. were kept in refrigerator at 4°C for further use.

The pathogen *Fusarium oxysporum* f.sp *betae* (*F. o. b.*)

The agriculture research center, plant pathology institute, Giza, Egypt, kindly gave a pure culture of *Fusarium oxysporum* f. sp. *betae* (*F. o. b*.), the causative agent of sugar beet wilt disease. Culture of the pathogen was maintained on Czapek´s dox agar medium plates and slants and were incubated at 28°C for 7 and then kept at 4°C until needed.

Screening the antifungal efficacy of the four tested *Trichoderma* spp. against *F. oxysporum* f.sp *betae* using the dual method

Based on the methodology outlined by **Yassin *et al*. (2021),** four species of *Trichoderma* (*T. harzianum, T. hamatum, T. galaucum* and *T. viridie*) were tested for their antifungal activities against *Fusarium oxysporum* f.sp *bet*ae. Five mm aseptically cut mycelial disc was put one centimeter from the edge of each 9 cm diameter petri dish with PDA media. The discs were taken from a 7-day-old *F. o. b.* culture. Also, on the other end of the same petri dish, five

millimeter discs of each used *Trichoderma* species were placed separately, one centimeter from the edge. *Trichoderma* spp. were tested using three replicate plates for each species. The control and all of the plates were kept at 25°C for five days for incubation. The following formula was used to calculate the percentage of growth inhibition after incubation:

I=A-B/A×100

Where I is the percentage of the inhibition of the mycelial growth of *F.o.b*, A is mycelial growth diameter of pathogen *F. o. b*. in control plate and B is mycelial growth diameter of pathogen in treatment plate for each *Trichoderma* species.

Screening the antifungal efficacy of the tested *Trichoderma* spp. against *F. oxysporum* f.sp *betae* using the disc method

Three mycelial discs each 5mm were taken from each of freshly cultures of the four tested *Trichoderma* spp. separately and inoculated into flasks each containing 100 ml of autoclaved PDB medium at pH7 Flasks were incubated for 5 days at 25°C **(You *et al*., 2016)**. After incubation, each culture of the two selected *Trichoderma* spp. was filtered separately using Whatman no. 1 filter paper, then filtrates were sterilized using a 0.22 µm millipore filters and mixed with unsolidified sterilized PDA medium at ratio 10% (v/v) and were poured into petri dishes. Untreated PDA medium was used for control plates. Five mm mycelial disc of *F. o. b.* was placed in the center of each PDA plate and were incubated at 28°C for 7 days. Three plates were used for each *Trichoderma* spp. Inhibition percentage of the mycelial growth diameters (cm) of *F. o. b*. by each of *Trichoderma* spp. were calculated to mycelial growth in control plates according the formula **(Zaki *et al.*, 2021)**.

I=A-B/A×100

Where I is the percentage of the inhibition of the mycelial growth of *F. o. b.,* A is mycelial growth diameter of pathogen *F. o. b*. in control plate and B is the mycelial growth diameter of pathogen in treatment plate for each *Trichoderma* species

Optimization of cultures conditions of the two selected most potent *T. harzianum* and *T. hamatum*

The cultures optimizations of *T. harzianum* and *T. hamatum* were carried out to evaluate several parameters to maximize the antifungal materials productivity against the pathogen *F. o. b.* The optimal result achieved by each factor was fixed for the subsequent experiment. These parameters included incubation periods (2, 4, 6 ,8 and 10 days), incubation temperatures (20, 25, 30 and 35°C), pH values (4, 5, 6,7 and

8), different carbon sources (glucose, fructose, maltose , sucrose and galactose) and different nitrogen sources (sodium nitrate, potassium nitrate, ammonium phosphate, malt extract, yeast extract and peptone). All Parameters were carried out separately using 250 ml conical flasks for each one, each flask containing 100 ml of autoclaved Czapek 's dox liquid medium at pH 7. The flakes were inoculated separately with 5mm from each of *T. harzianum* and *T. hamatum* separately at each factor. All flakes of each factor were incubated. After incubation, each parameter of each culture of the two selected *Trichoderma* spp. was filtered and the mycelium was washed by sterile distilled water twice and dried at 60°C in an oven for determination of the mycelial dry weight (mg /100).

Antifungal activities of the most effective filtrates of *T. harzianum, T. hamatum* individually and in combination under the optimized conditions against *F. oxysporum* f.sp *betae* using disc method

Each of the two most effective *Trichoderma* spp. was cultured to obtained filtrate under the optimized conditions **(You *et al*., 2016)**. Mycelial discs (5mm) was taken from 7 days old cultures of each of the two selected *Trichoderma* spp. separately and inoculated into conical flasks (250 ml) containing 100 ml autoclaved Czapex's dox liquid medium at pH 6 contained yeast extract and sucrose as nitrogen and carbon sources respectively and flasks were incubated for 8 days at 30°C**.** Following the incubation period, the two cultures of *Trichoderma* spp. were filtered separately using Whatman no. 1 filter paper. The resulting filtrates of the two *Trichoderma* spp. were then sterilized using 0.22 µm millipore filters. They were then combined with PDA medium and solidified at a 10% (v/v) concentration. For the control, the same ratio of uninoculated Czapex's dox agar to PDA was used. For the two *Trichoderma* spp. filtrates and the control plates, a 5 mm mycelial disc of *F. o. b*. was put in the middle of each PDA plate and incubated at 28°C for 5 days. In order to determine the percentage of inhibition of mycelial growth of *F. o. b*. compared to the control, the following formula was used **(Zaki *et al*., 2021)**

$$I=A-B/A\times100.$$

Where I is the percentage of the inhibition of the mycelial growth of *F. o. b*.,A is mycelial growth diameter of pathogen *F. o. b*. in control plate and B is mycelial growth diameter of pathogen in treatment plate for each *Trichoderma* species.

Molecular identification of *F. oxysporum* f.sp *betae* and the two selected *T. harzianum, T. hamatum* for confirmation the identification

After being incubated for 7 days at 25°C, the two effective *Trichoderma* spp. were grown on Czapek's yeast agar (CYA) plates **(Pitt and Hocking, 2009).** A tiny amount of fungal mycelium was taken from each sample, mixed with 100 milliliters of distilled water, heated at 100°C for 15 minutes, and then stored at -70°C. We have supplied SolGent with samples. **(Daejeon, South Korea)** perform each step of the process, beginning with DNA extraction and ending with DNA sequencing. The SolGent purification bead was used to extract and isolate the fungal DNA. Primer sets ITS1 and ITS4 were used to amplify the ribosomal DNA's internal transcribed spacer (ITS) region (5' - TCC GTA GGT GAA CCT GCG G - 3') and (5' - TCC TCC GCT TAT TGA TAT GC - 3'), respectively. The ABI 9700 thermal cycler was used to conduct the amplification. Using Solgent EF-Taq, the following steps were taken to create the PCR mixtures: 10 millimoles of dNTP (T), 2.5 microliters of 10X EF-Taq buffer, 1 microliter of primer (R-10p), 0.25 microliters of EF-Taq (2.5U), 1 microliter of primer (F-10p), 1 microliter of template, and 25 microliters of DW. In order to amplify the target DNA, a PCR reaction was performed with the following parameters: first, a 15-minute denaturation at 95°C; second, 20 seconds of annealing at 50°C; third, 1 minute of extension at 72°C; and last, 5 minutes of extension at 72°C. Following this, the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) was used to prepare the PCR products for sequencing. The separated PCR products were validated by electrophoreses on a 1% agarose gel with the use of size markers. The bands were subsequently eluted and sequenced. Both the sense and antisense routes of sequencing were performed on each sample using the identical primers and ddNTPs (big dye). From the sequencing data, contigs have been generated using the CLCBio Main Workbench software. The acquired sequences have been sent for additional examination using BLAST, which can be found on the NCBI website. Together with sequences obtained from the GenBank database (http://www.ncbi.nlm.nih.gov), the sequences were run through the Clustal W analysis in MegAlign version 5.05 (DNASTAR Inc., Madison, Wisconsin, USA) for the phylogenetic analysis **(Thompson *et al.*, 1994**).

Extraction the antifungal materials from the two most potent *T. harzianum* and *T. hamatum*

Mycelial disc 5mm was transferred from 7days old cultures of each of *T. harzianum* and *T. hamatum* and was inoculated separately into flasks (250ml) each containing 50ml of autoclaved PDB and adjusted at pH 6 then incubated under the optimized conditions at 30°C for 8 days. Following incubation, the culture filtrate of each species of *Trichoderma* was separated and collected using Whatmann No. 1 filter paper, followed by centrifugation at 9000 rpm for 15 minutes, and ethyl acetate was used as a solvent to extract the antifungal components. The extracts were further concentrated using rotary evaporator to evaporate the solvents **(Zaki *et al.*, 2021)** .

Characterization of the extracted antifungal materials of the most potent *T. harzianum* and *T. hamatum* using gas Chromatography–mass spectrometry (GC-MS)

According to the method outlined by **Shahiri Tabarestani *et al*., (2016)**, the antifungal components isolated from the two most potent *T. harzianum* and *T. hamatum* were analyzed using gas chromatography–mass spectrometry (GC–MS). The tests were conducted using an HP-5MS fused silica capillary column (30 m, 0.25 mm i.d., 0.25 mm film thickness) coupled to an Agilent 8890 gas chromatography system and a mass spectrometer (Agilent 5977B GC/MSD). For the first two minutes, the oven was programmed to maintain a temperature of 40°C. The program called for a 5°C/min increase from 40 to 200°C, a 25°C/min increase from 200°C to 260°C, and a 25-minute hold at 260°C. A flow rate of 1.1 mL/min was used for the helium carrier gas. At 250°C, the injection was heated. At 70 eV, the electron impact mode (EI) was used to acquire mass spectra that scanned from 39 to 500 amu in m/z. Recognizing the peaks required comparing the isolated ones to data stored in the NIST mass spectra resource.

Statistical Analysis

Analysis of variance (ANOVA) was used in the data analysis performed in SPSS program. The average differences were contrasted with the less significant difference (LSD) test set at $p < 0.05$.

Results

Screening the antifungal efficacy of the tested *T. harzianum, T. hamatum, T. galaucum* and *T. viridie* against *F. oxysporum* f. sp *betae* using the dual method

The results presented in **Table (1) and Photo (1)** showed that all the tested *Trichoderma* species (*T. harzianum*, *T. hamatum*, *T. viridie* and *T. galaucum*) recorded antagonistic effect against *F.o.b.* where the inhibition percentage of the mycelial growth of *F.o.b.* were 72.53,

71.30, 68.33 and 67.78 % respectively. The highest antagonistic effects were recorded using *T. harzianum* followed by *T. hamatum* against *F.o.b*. with inhibition percentage of mycelial growth 72.53 and 71.30 % respectively.

Table (1): Screening the antifungal efficacy of the tested *T. harzianum, T. hamatum, T. viridie* and *T. galaucum* against the pathogen *F.oxysporum* f.sp *betae* using dual method

Different *Trichoderma* Spp.	Mycelial growth diameters (cm) of *F.o.b*	Percentage (%) mycelial inhibition of *F.o.b*
T.harzianum	2.5±0.2	72.53
T.hamatum	2.6±0.3	71.30
T.viridie	2.8±0.3	68.33
T.galaucum	2.9±0.1	67.78
Control	9±0.1	0.00

Significance at P value < 0.05

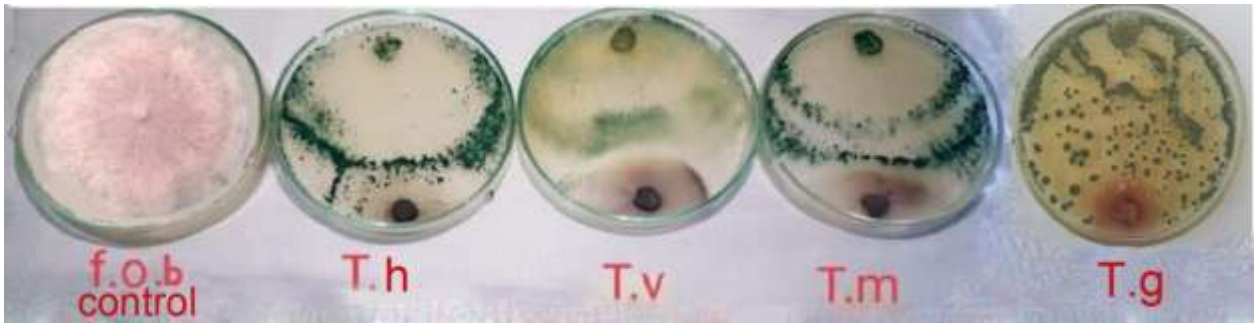

Photo (1): Dual culture assay of *T. harzianum* (*T. h*), *T. viridie* (*T. v*) *T. hamatum* (*T. m*) and *T. galaucum* (*T. g*) against *F. oxysporum* f. sp. *betae* (*F. o. b.*).

Screening the antifungal efficacy filtrates of the four tested *Trichoderma* spp. against *F. oxysporum* f. sp *betae* using the disc method

Table (2) illustrated that the effect of the culture filtrates of the different tested *Trichoderma* spp. against *F.o.b.* using the disc method. The results recorded that cultures filtrates of all the tested *Trichoderma* spp. exhibited significant percentages (%) inhibition to mycelial growth of *F. o. b*. where the % of mycelial growth inhibition were 83.50, 80.00, 74.10 and 65.90% respectively. The highest inhibitory effect on the mycelial growth diameters of *F. o. b*. were recorded by *T. harzianum* and *T. hamatum* where the mycelial growth diameters were reduced to 1.4 and 1.7 cm respectively, with percentages inhibitions 83.50 and 80.00% respectively comparing to the control. While *T. viridie* and *T. galaucum*

recorded lesser inhibitory effect on *F.o.b*. where the mycelial growth diameters were 2.2 and 2.9 cm with percentages inhibition 74.10 and 65.90% respectively.

Table (2): Screening the antifungal efficacy of the four tested *Trichoderma* spp. against the pathogen *F. oxysporum* f. sp. *betae* using the disc method

Different *Trichoderma* Spp. filtrates	Mycelial growth diameters (cm) of *F.o.b*	Percentage (%) mycelial inhibition of *F.o.b*
T.harzianum	1.4 ±0.2	83.50
T.hamatum	1.7±0.3	80.00
T.viridie	2.2±0.1	74.10
T.galaucum	2.9±0.2	65.90
Control	8.5±0.1	0.00

Significance at P value < 0.05

Optimization of cultures conditions of the two most potent selected *T. harzianum* and *T. hamatum* against *F. oxysporum* f.sp *betae*

Effect of the different incubation periods on the mycelial dry weight

The results in **Fig. (1)** showed that the mycelial dry weight of both *T. harzianum* and *T. hamatum* increased gradually with increasing incubation periods from the two days to 8 days where the mycelial dry weight was 90.30, 127.30, 178.30, 231.60 and 56.00, 92.00, 144.00, 181.00 mg/100ml respectively. The highest mycelial dry weight was recorded at 8 days, the optimal incubation period for both *T. harzianum* (231.60 mg/100ml) and *T. hamatum* (181.00 mg/100ml) followed by incubation period at 10 days where the mycelial dry weight of both *T. harzianum* and *T. hamatum* were 217.30 and 178.30 mg/100ml.

Effect of the different incubation temperatures on the mycelial dry weight of *T. harzianum* and *T. hamatum*

Figure (2) illustrated the effect of different temperatures (20, 25, 30 and 35°C) on the mycelial dry weight of *T. harzianum* and *T. hamatum.* The results recorded increasing in mycelial dry weight of *T. harzianum* with increasing incubation temperature until reached to the optimal temperature 30°C where the highest mycelial dry weight was 223.60 mg/100ml. While the dry weight of *T. hamatum* also increased with the increasing incubation temperature until reached to the maximum weight 157.60 mg/100ml at 35°C (the optimal incubation temperature).

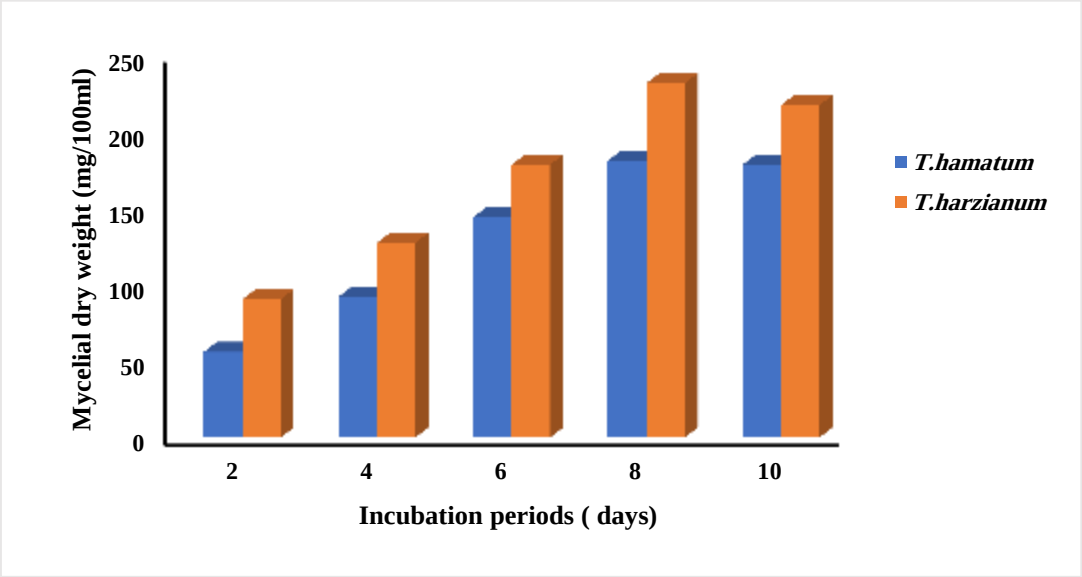

Fig. (1): Effect of the different incubation periods on the mycelial dry weight of *T. harzianum* and *T. hamatum*

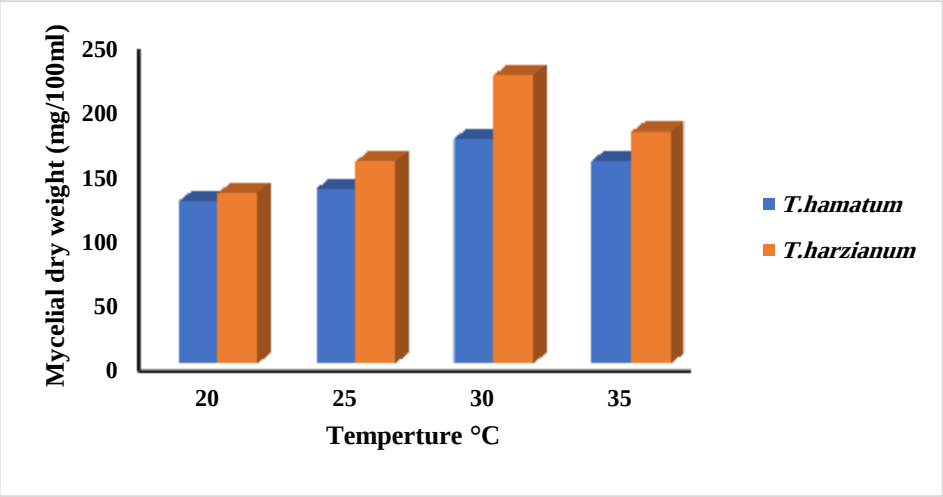

Fig. (2): Effect of the different temperatures on the mycelial dry weight of *T. harzianum* and *T. hamatum*

Effect of the different carbon sources on the mycelial dry weight of *T. harzianum* and *T. hamatum*

Figure (3) showed the effect of different carbon sources (galactose, fructose, glucose, maltose and sucrose) on the mycelial dry weight of *T. harzianum* and *T. hamatum.* The results exhibited that the highest mycelial dry weight of both *T. harzianum* and *T. hamatum* were recorded with using sucrose as carbon source where the mycelial dry weight were 179.60 and 131.30 mg/100ml respectively, followed by galactose (150.60 mg/100ml), glucose (146.00 mg/100ml), and maltose (141.00 mg/100ml) for *T. harzianum* and maltose (119.30 mg/100ml), glucose (105.60 mg/100ml), galactose (101.60 mg/100ml) for *T. hamatum*. The lowest mycelial dry weight for both *Trichoderma* spp. were recorded with using fructose as a carbon source **(Fig. 3)**.

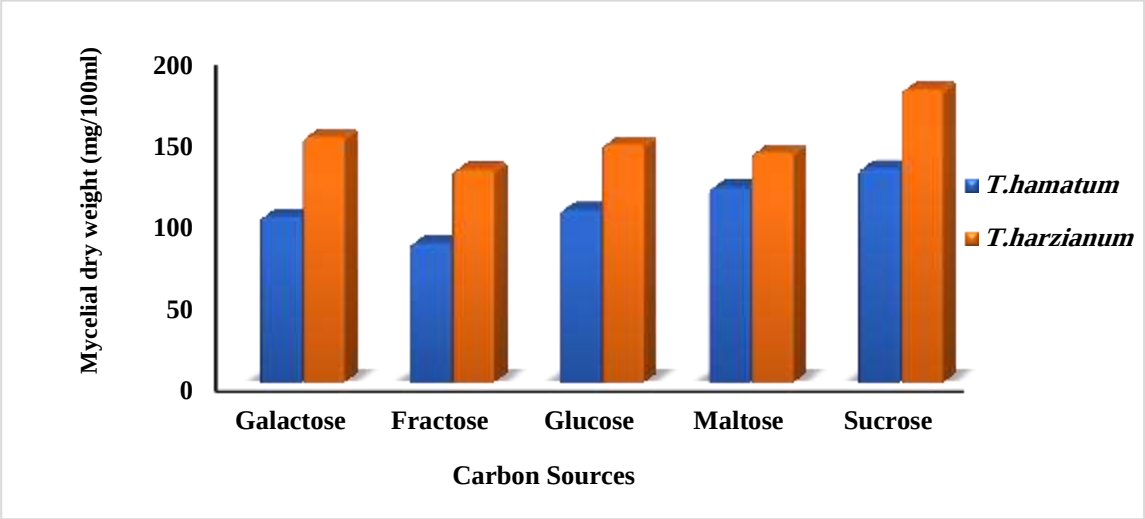

Fig. (3): Effect of the different carbon sources on the mycelial dry weight of *T. harzianum* and *T. hamatum*

Effect of the different nitrogen sources on the mycelial dry weight of *T. harzianum* and *T. hamatum*

The data in **Fig. (4)** showed the effect of the different nitrogen sources (yeast extract, peptone, malt extract, amonium phosphate, KNO_3 and $NaNO_3$) on the mycelial dry weight of *T. harzianum* and *T. hamatum.*, the results showed that the highest mycelial dry weight of both *T. harzianum* and *T. hamatum* were recorded with using yeast extract where the mycelial dry weight were 213.00 and 164.00mg/100ml respectively, followed by peptone and malt extract with mycelial dry weight of 191.00, 147.30 mg/100ml and 186.00, 140.00 mg/100ml respectively. While the lowest mycelial dry weight of both

Trichoderma spp. was obtained with KNO_3 followed by $NaNO_3$ and amonium phosphate.

Effect of the different medium pH values on the mycelial dry weight of *T. harzianum* and *T. hamatum*

Figure (5) showed that, the effect of different pH values (4, 5, 6, 7 and 8) on the mycelial dry weight of *T. harzianum* and *T. hamatum.* The recorded results indicated that the highest mycelial dry weights of both *T. harzianum* and *T. hamatum* were obtained at pH 6 (243.00 and 189.00 respectively) followed by pH7 with mycelial dry weight of 201.60 and 178.60 respectively. While the lowest mycelial dry weights for both tested *Trichoderma* spp. were recorded at pH 5, 8 and 4 **(Fig.5)**.

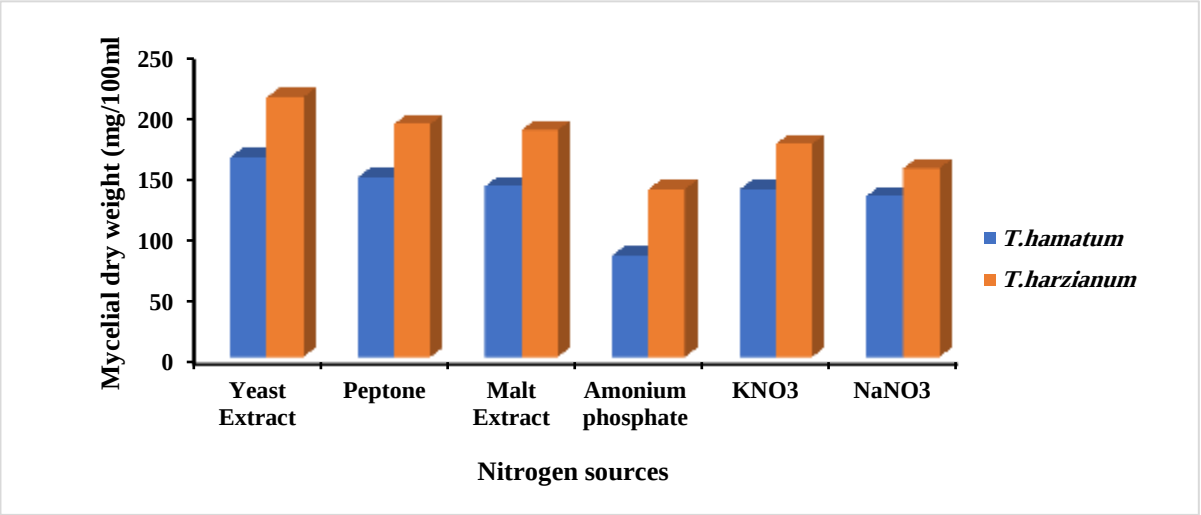

Fig. (4): Effect of the different nitrogen sources on the mycelial dry weight of *T. harzianum* and *T. hamatum*

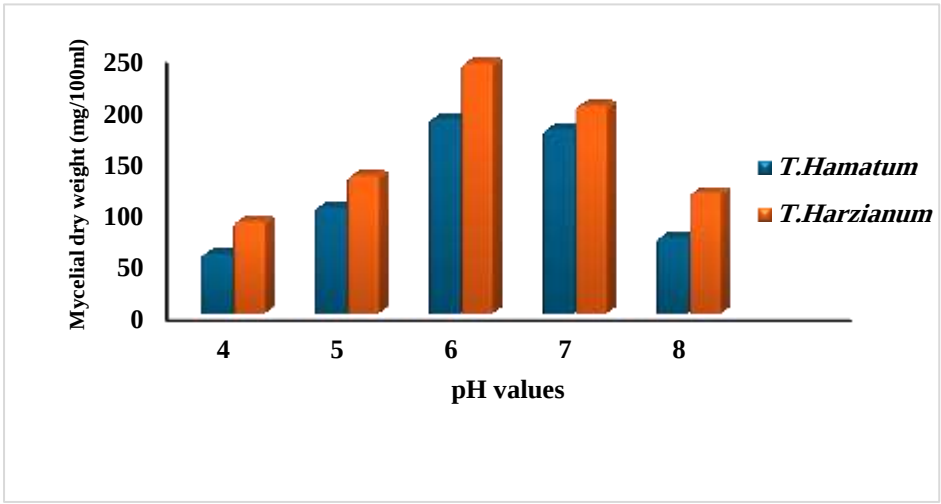

Fig. (5): Effect of the different medium pH values on the mycelial dry weight of *T. harzianum* and *T. hamatum*

Antifungal activities of the most potent *T. harzianum* and *T. hamatum* filtrates individually and in combination under the optimized conditions against *F. oxysporum* f.sp *betae* using the disc method

Results in **Table (3)** showed the antifungal activities of *T. harzianum and T. hamatum* filtrates individually and in combination which were prepared under the optimized conditions. The results recorded higher inhibitory effect of *T. harzianum* and *T. hamatum* against *F. o. b.* more than before optimization, with using the disc method, where the reduction in diameters of mycelial growth of *F. o. b.* were 1.6 and 1.3 cm with percentage inhibitions 85.50 and 82.20 % respectively comparing to control 9.0 cm. The results also revealed that the using combination between the two filtrates of *T. harzianum* and *T. hamatum* were more effective and recorded the highest inhibitory effect against *F. o. b* where the mycelial growth diameter was reduced to 1.2 cm with percentage inhibition reached to 86.70 %.

Table (3): Antifungal activities of the most potent *T. harzianum* and *T. hamatum* filtrates individually and in combination under the optimized conditions against *F. oxysporum* f. sp. *betae* using the disc method

Different *Trichoderma* spp. filtrate	Mycelial growth diameters(cm) of *F.o.b*	Percentage (%) mycelial inhibition of *F.o.b*
T.harzianum	1.3±0.2	85.50
T.hamatum	1.6±0.3	82.20
T.harzianum+T.hamatum	1.2±0.2	86.70
Control	9±0.1	0.00

Significance at P value < 0.5

Molecular identification of *F. oxysporum* f.sp *betae* and the two selected *T. harzianum* and *T. hamatum*

The phylogenetic tree shown in **Fig. (6.a)** uses the ITS sequences of rDNA to show how the strain of *Fusarium oxysporum* f.sp. *betae* used in this work (*F. o. b.*AUMC16284, arrowed) compares to comparable strains in GenBank. Phylogenetic tree based on ITS sequences of rDNA of the used fungal sample (*Trichoderma asperellum* AUMC16285, arrowed) and closely related strains accessed from GenBank. The *Fusarium oxysporum* f.sp. *betae* strain showed 99.63% - 100% similarity and 99% - 100% coverage with several strains of this species. **Fig. (6.b)** illustrates this structure. **Fig. (6.c)** shows a phylogenetic tree based on ITS sequences of rDNA of the fungal sp. in this study (*Trichoderma harzianum*

AUMC16286, arrowed), which exhibits 100% identity and 100% coverage with multiple strains of the same species. These strains are closely related to each other and were retrieved from GenBank.

The result of *Fusarium* sp. sequencing indicated that the *Fusarium* sp. isolate was *Fusarium oxysporum* f.sp *betae* . that was identified and recorded in gene bank with accession number of PP410242 and the results of *Trichoderma hamatum* which was identified by *Trichoderma asperellum* and recorded in the gene bank with acc. no. of PP410284. The results of the other *Trichoderma* sp. strain were *Trichoderma harzianum* that was identified and recorded in the gene bank with acc. no. of PP410286.

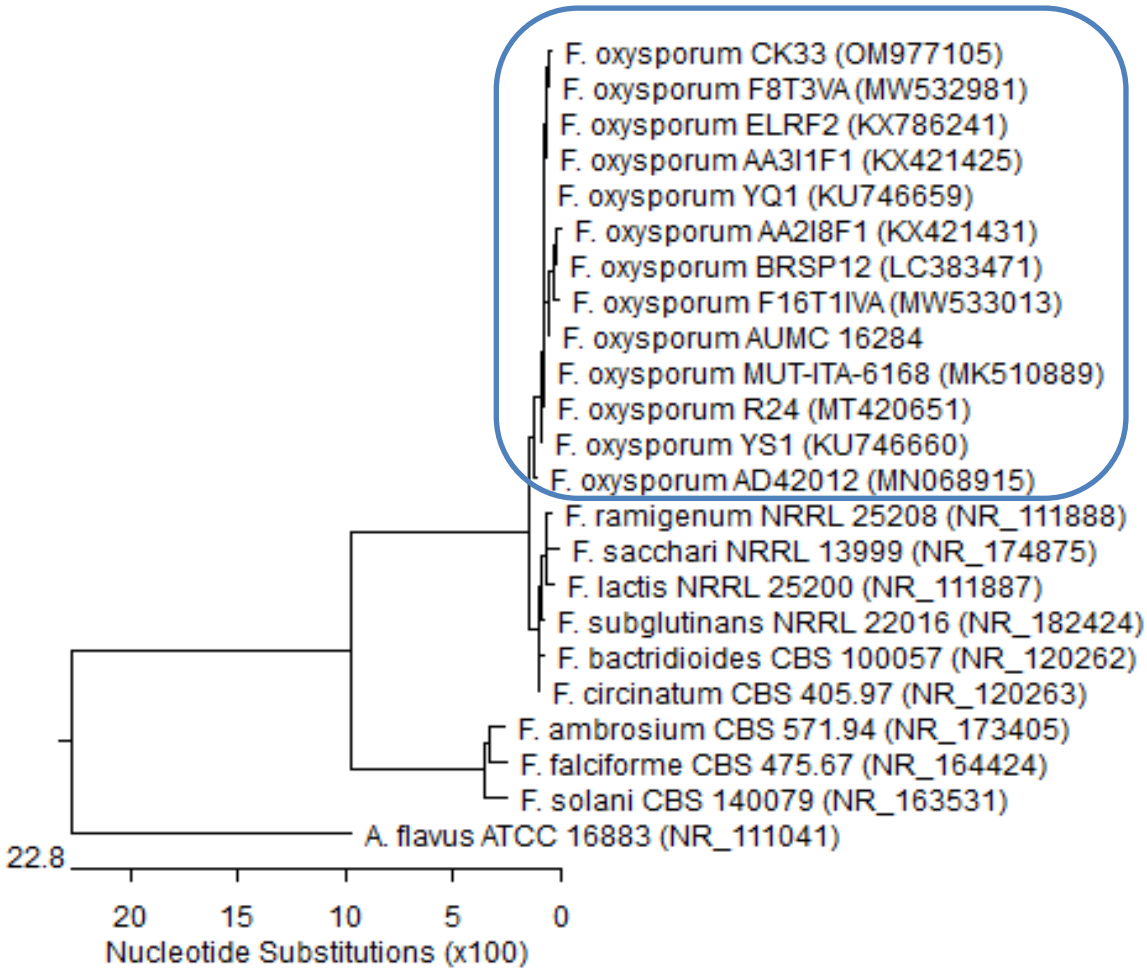

Fig. (6.a): Phylogenetic tree based on ITS sequences of rDNA *F. oxysporum* f.sp. *betae* (*F. o. b* AUMC16284, arrowed) aligned with closely related strains accessed from the GenBank. This strain showed 99.63% -100 % identity and 99% -100% coverage with several strains of the same species with accession no. PP410242

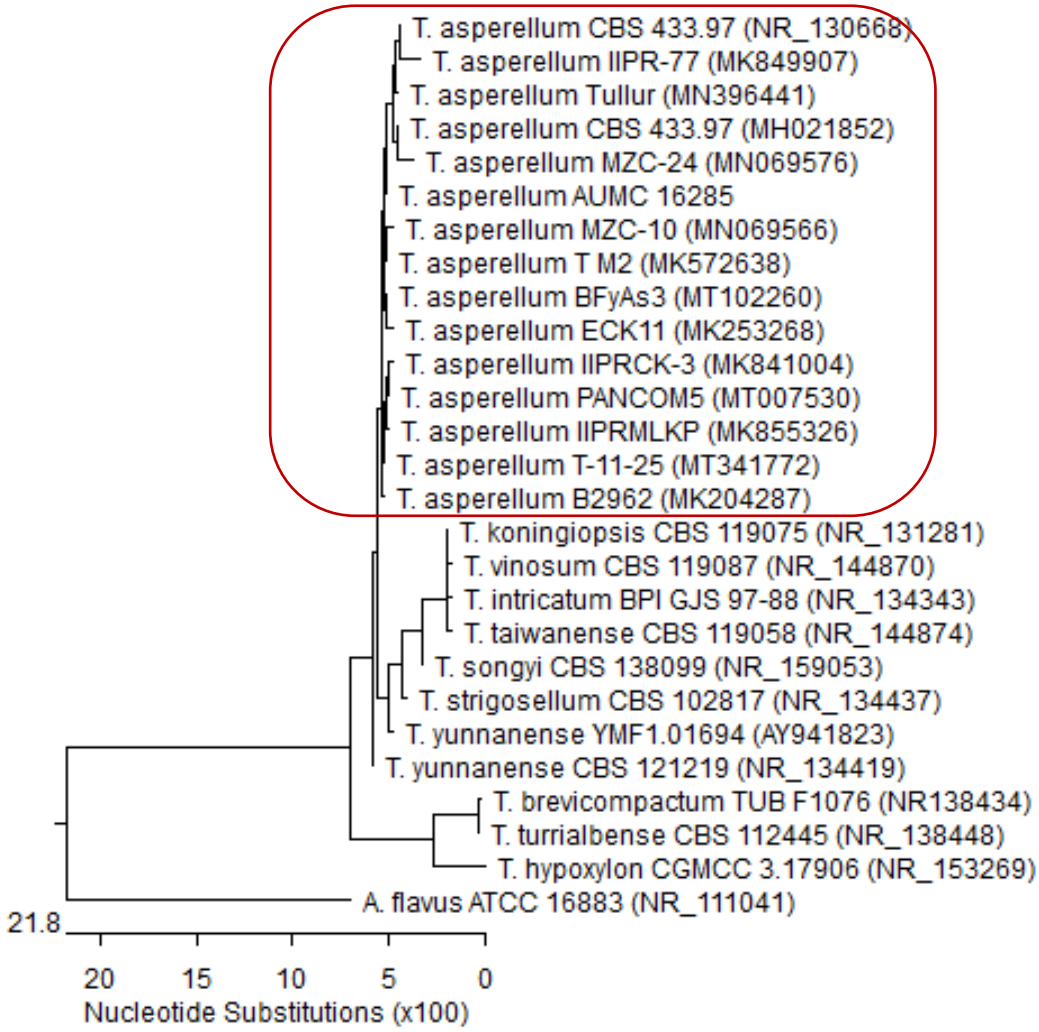

Fig. (6.b): Phylogenetic tree based on ITS sequences of rDNA (*Trichoderma asperellum* AUMC16285, arrowed) aligned with closely related strains accessed from the GenBank. This strain showed 100 % identity and 90% - 100% coverage with several strains of the same species with accession no. PP410284

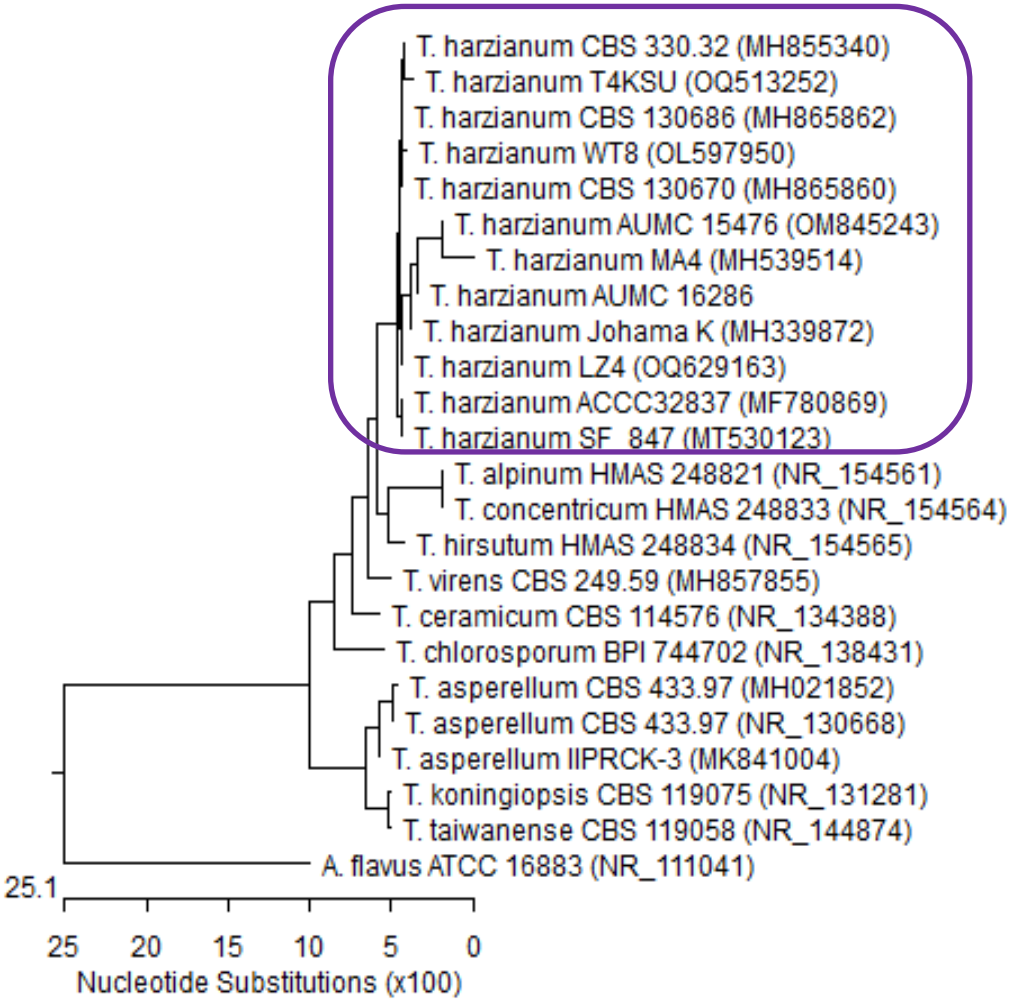

Fig. (6.c): Phylogenetic tree based on ITS sequences of rDNA of (*Trichoderma harzianum* AUMC16286, arrowed) aligned with closely related strains accessed from the GenBank. This strain showed 100 % identity and 100 % coverage with several strains of the same species with accession no. of pp 410286

Characterization of the antifungal materials of the most effective *T. harzianum* and *T. hamatum* by gas Chromatography–mass spectrometry (GC-MS)

By using GC-MS analysis the antifungal components that impeded the growth of *F. oxysporum* f.sp *betae* were identified in the extracts of *T. harzianum* and *T. hamatum.* The results demonstrated that both *Trichoderma* spp. produced a wide variety of chemicals with strong antimycotic activity where the data of GC-MS analysis recorded that the antifungal materials produced by *T. harzianum* were palmitic acid, oleic acid, pentadecanoic acid, and trans-13-octadecenoic acid as illustrated in **Table (4 a) and Fig. (7a).** While the compounds produced by *T. hamatum* were**,** palmitic acid, Oleic Acid, Pentadecanoic acid, linoleic acid and trans-13-Octadecenoic acid **(Table 4 b and Fig.7 b).**

Table (4 a): Characterization of the antifungal materials of *T. harzianum* using gas Chromatography–mass spectrometry (GC-MS)

Peak	RT	Area Sum %	Components of *T.harzianum* antifungal materials	Biological activity
1	6.326	2.69	Furfural	-
2	12.648	1.11	Benzeneacetaldehyde	Antioxidant
3	13.889	2.93	2-Furfurylfuran	-
4	20.727	1.83	* n-Decanoic acid	Antimicrobial activity
5	24.349	2.19	6-Pentyl-2H-pyran-2-one	-
6	26.833	2.6	(-)-Globulol	-
7	27.153	3.8	Bulnesol	-
8	27.531	0.99	Bisabolol oxide II	-
9	27.599	2.56	Retinal	Antioxidant
10	28.847	1.81	cis-5,8,11,14,17-Eicosapentaenoic acid	Antimicrobial activity
11	29.116	0.97	Humulenol	-
12	29.43	3.38	γ-Gurjunenepoxide-(2)	Antioxidant activity
13	29.883	5.24	α-Costol	-
14	30.14	1.89	Cedr-8-en-13-ol	-
15	30.604	3.06	trans-Nuciferol	-
16	30.895	0.98	Dehydroxy-isocalamendiol	Antioxidant activity
17	31.273	6.04	Isoaromadendrene epoxide	Antioxidant activity
18	32.211	1.04	2-Phenoxyethyl phenyl ether	-
19	32.864	1.47	Ethyl iso-allocholate	-
20	33.493	2.87	Diisobutyl phthalate	-
21	33.665	0.92	2-Methyl-1-hexadecanol	Antioxidant activity
22	33.728	1.85	6-Undecylamine	-
23	34.494	2.78	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	-
24	34.798	5.33	Palmitoleic acid	Antimicrobial activity
25	35.072	19.76	*Palmitic acid	Antimicrobial activity
26	35.278	2.48	Palmitic acid vinyl ester	Antimicrobial activity
27	35.685	0.69	Isopropyl palmitate	-
28	35.925	0.87	cis-13-Eicosenoic acid	Antioxidant activity
29	36.657	5.87	*Oleic Acid	Antioxidant activity
30	36.686	6.38	cis-Vaccenic acid	-
31	36.8	2.69	*trans-13-Octadecenoic acid	Antimicrobial activity
32	40.239	0.91	Phthalic acid, di(2-propylpentyl) ester	-

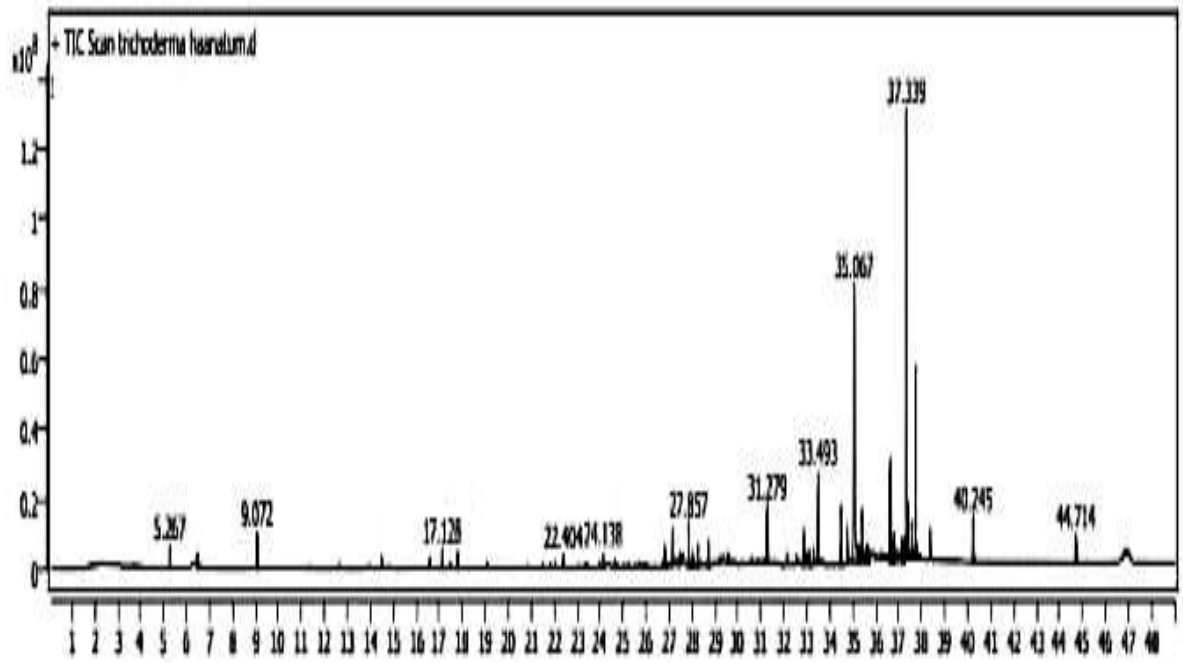

Fig. (7 a): GC/MS of *T. harzianum* antifungal materials

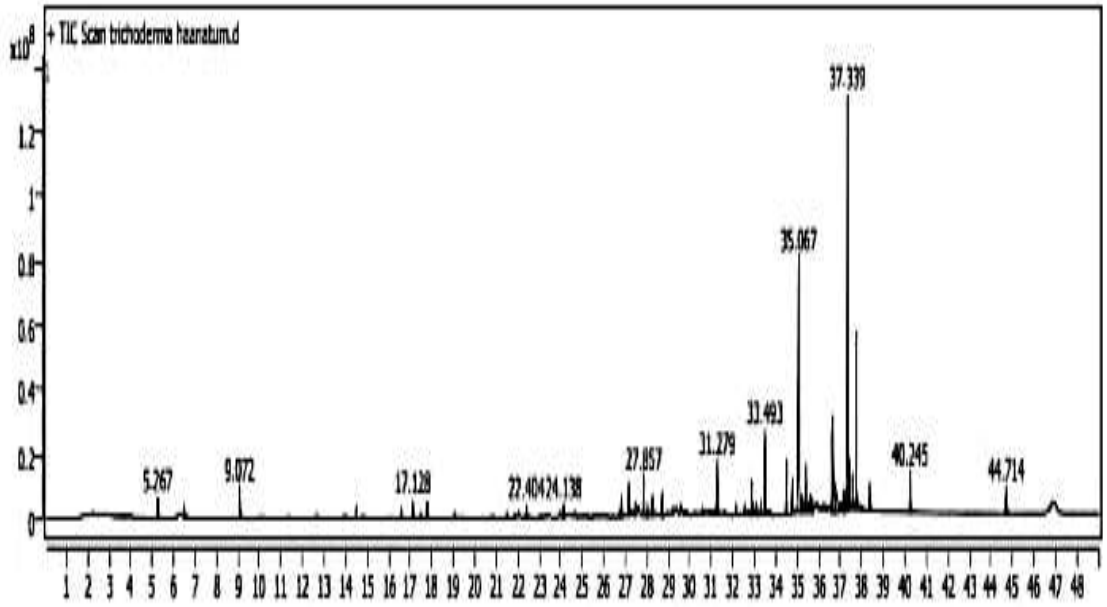

Fig. (7 b): GC/MS of *T. hamatum* antifungal materials

Table (4 b): Characterization of the antifungal materials of *T. hamatum* by gas Chromatography–mass spectrometry (GC-MS)

Peak	RT	Area Sum %	Components of *T.hamatum* antifungal materials	Biological activity
1	6.474	0.69	Furfural	-
2	9.072	1.51	Methyl N-hydroxybenzenecarboximidoate	Antioxidant activity
3	17.128	0.92	Nona-3,5-dien-2-one	-
4	17.804	0.9	2,5-Dimethylbenzaldehyde	Antimicrobial activity
5	22.404	0.8	Methyl cinnamate	-
6	24.138	0.66	Aromadendrene oxide-(2)	-
7	26.816	1.34	(-)-Globulol	-
8	27.159	1.98	Bulnesol	-
9	27.457	0.88	cis-Z-α-Bisabolene epoxide	Antioxidant activity
10	27.857	2.15	cis-5,8,11,14,17-Eicosapentaenoic acid	Antimicrobial activity
11	28.035	0.66	Caryophyllene oxide	-
12	28.263	1.08	Isoshyobunone	-
13	28.715	1.17	tau.-Cadinol	-
14	31.279	3.81	Tetradecanoic acid	Antimicrobial activity
15	32.87	1.71	trans-Geranylgeraniol	-
16	33.099	0.75	Methyl 2,5-octadecadiynoate	Antimicrobial-Antioxidant activity
17	33.305	0.98	*Pentadecanoic acid	Antimicrobial activity
18	33.493	4.28	Diisobutyl phthalate	-
19	34.495	2.47	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	-
20	34.764	1.95	Palmitoleic acid	Antimicrobial activity
21	35.067	18	*Palmitic acid	Antimicrobial activity
22	35.244	1.49	Palmitic acid vinyl ester	Antimicrobial activity
23	35.405	2.7	Verticiol	-
24	35.593	1.33	Methyl glycocholate, 3TMS derivative	-
25	35.691	1.16	Isopropyl palmitate	-
26	36.612	2.83	*Linoleic acid	Antimicrobial activity
27	36.646	5.99	*Oleic Acid	Antimicrobial activity
28	36.795	3.08	*trans-13-Octadecenoic acid	Antimicrobial activity
29	37.121	1.08	Ethyl iso-allocholate	-
30	37.23	1.44	Ethyl iso-allocholate	-
31	37.339	16.07	1-Hydroxy-3-methylanthraquinone	--
32	37.402	1.77	Sclareol	-
33	37.562	1.22	Tributyl acetylcitrate	-
34	37.728	5.57	Pimara-7,15-dien-3-one	-
35	38.357	1.38	4,5-Dihydroxy-2-methylanthraquinone	-
36	40.245	2.09	Phthalic acid, di(2-propylpentyl) ester	Antimicrobial-antioxidant activity
37	44.714	2.1	Squalene	-

Discussion

In many countries, sugar beets are among the most important crops for producing sugar. More sugar beet production is required to fulfill Egypt's demands, as the country has a high sugar consumption rate. The most devastating soil-borne fungal disease that impacts sugar beets is Fusarium wilt, which is caused by *Fusarium oxysporum* f.sp. *betae.* Mycelium and chlamydospores of *Fusarium oxysporum* f. sp. *betae* can persist in soil and plant detritus for a long time. Under the favorable conditions, the fungus penetrates the roots of sugar beets and gains entry to the vascular system, where it moves up the plant and causing foliar symptoms. Additionally, the fungus functions as a "plug" blocking the flow of water in the vascular tissue of sugar beet and subsequently, causes wilting of the plant. The wilt symptoms caused by *Fusarium oxysporum* f.sp. *betae* are yellowing, chlorosis, wilting, and necrosis of the leaves **(Hill *et al.*, 2010)**, infected plants systemically cannot be cured and must be destroyed as soon as possible **(Summerell *et al.*, 2011).** Biological control, is a renaissance in the study of microbial balance for the management of soil-borne plant illnesses, can lead to a more effective agricultural system. One of the greatest bioagents for biological control is the genus *Trichoderma,* which is proven to be efficient against a variety of foliar and soil pathogens **(Chen *et al.*, 2021).** *Trichoderma* spp. have the ability to biocontrol plant diseases because of their intricate interactions with them, which can involve parasitization, the secretion of antibiotics, antifungal materials or competition for nutrients and space. *Trichoderma* is a biological fungus that is frequently employed to manage plant pests **(Haouhach *et al.*, 2020; Wang *et al.*, 2022). Tyśkiewicz *et al.*, (2022)** researched the use of *Trichoderma* as a tool for disease management in plants. Both *T. viride* and *T. harzianum* suppress plant pathogens, albeit to varying degrees.

The present work used the dual and disc methods to test the antifungal efficacy of four species of *Trichoderma*: *T. harzianum, T. hamatum, T. viridie* and *T. galaucum.* against *Fusarium oxysporum* f. sp. *betae*. Each of the four species of *Trichoderma* that were tested showna strong antagonistic action against the pathogen *F.o.b.*, according to the recorded results. Out of the four *Trichoderma* spp. *T. harzianum* and *T. hamatum* recorded the highest inhibitory effect against *F.o.b.* with mycelial inhibition percentage reached to 72.53 and 71.30% respectively with using

either the dual or disc methods. Our results are in agreement with those of **Rudresh *et al.*, (2005)**, their study on the antibacterial activity of culture filtrates of *T. harzianum* against the *F. oxysporum* strain where they found that the mycelial inhibition rates reached to 78.5%. **Alvarez-Garcia *et al.*, (2020)** further demonstrated that *T. harzianum* culture filtrates exhibited a 76.27% inhibition rate in the mycelial development of *Fusarium* spp. The findings presented by **Tomah *et al.*, (2020)** reported that 77.8% of fungal pathogen growth was slowed by *Trichoderma citrinoviride.* Also, the results were obtained by **Yogalakshmi *et al.*, (2021)** was in agreement with the current study that investigated the bio-efficacy of fungal bio agents from fifteen different *Trichoderma* species against *Fusarium oxysporum* f.sp. *lycoperesici*, the causing pathogen of tomato wilt disease. Four of the *Trichoderma* species had a more potent antagonistic effect on the tomato pathogens than the others. And also **El-Sobky *et al.*, (2019)** found that the five phytopathogens: Sclerotium spp., Alternaria alternata, Rhizoctonia solani, and *Fusarium oxysporum* f.sp. betae were all inhibited in their mycelial growth by the four Trichoderma strains that were identified.

Research on the use of *Trichoderma* spp. for disease management in plants conducted on a global scale. *Fusarium*, *Rhizoctonia*, *Botrytis*, and 29 other plant pathogenic fungal species (from 18 genera) are inhibited to varying degrees by *T. viride* and *T. harzianum. Trichoderma* species were successful in controlling a wide variety of phytopathogenic fungi, such as *Pythium ultimum, Rhizoctonia solani, Fusarium oxysporum* f.sp *betae*, *Botrytis cinerea, Sclerotinia sclerotiorum, Colletotrichum* species, and *Pseudocercospora* species **(Al-Askar *et al.*, 2021; Degani and Dor, 2021; Andrade-Hoyos *et al.*, 2022; Zhang *et al.*, 2022).**

Optimization of cultures conditions of the two selected most effective *T. harzianum* and *T. Hamatum* were carried out in the present study for maximizing the antifungal materials production by the two selected *Trichoderma* spp. , the results showed that the optimal conditions of each of the two *Trichoderma* spp. were incubation temperatures at 30°C, 8 days incubation, pH6, ntrogen and carbon sources were yeast extract and sucrose respectively. The study of antifungal activities of the filtrates of each of the two selected *Trichoderma* spp. and in combinationof both of the two filtrates which prepared under the optimized conditions showed highly increasing inhibition in the mycelial growth of *F.o.b.* reached to 85.50, 82.20% respectively and 87.70 %

in combination of the two *Trichoderma* spp. filtrates. **Zehra *et al.*, (2017)** reported that *T. harzianum* was found to be the most effective *Trichoderma* spp. when tested against *Alternaria alternata* and *F. oxysporum* under a variety of environmental circumstances, including temperature and pH. Additionally, **Yao *et al.*, (2023)** investigated the mechanisms of competition, antagonism, antibiosis, and mycoparasitism that *Trichoderma* uses to control fungal phytopathogens and nematode diseases. They also investigated at the mechanisms that *Trichoderma* uses to promote plant growth and induce systemic resistance in plants.

In the present work, identification of *F. oxysporum* f.sp *betae*, *T. harzianum* and *T. hamatum* were confermed by molecular identification based on internal transcribed spacer (ITS) sequences of rDNA genes and phylogenetic analysis which showed that *F. oxysporum* f.sp *betae* exhibited similarity 99% to *F. oxysporum* f.sp. *betae*, and recorded in the gene bank with accession no. PP410242, *T. harzianum* exhibited similarity 100% to *T. harzianum* with accession no. PP410286 in the gene bank. While *Trichoderma hamatum* identified as *Trichoderma asperellum* not *Trichoderma hamatum* where *T. hamatum* exhibited similarity 100 % to *T. asperellum* with accession no. PP410286 this was in agreement with **Abou Zeid and Mahmoud (2012),** who recovered *Trichoderma* spp. from *rhizosphere* soil samples collected from faba bean plants in the Fayoum governorate of Egypt. Morphological features were initially used to identify *Trichoderma hamatum*. Three markers were compared to the Gene Bank Database using blast after PCR amplification and two-way sequencing. The three marker sequences that were compared showed a perfect match with the strain *Trichoderma asperellum* sequences that were released in the Gene Bank: ITS (1+2), EF1, and Act. "Phylogenetic analysis could be used to construct a phylogenetic tree."

The GC-MS analysis of ethyl acetate extracts of the two most effective *T. harzianum* and *T. hamatum* were carried out to determine the antifungal materials. The results indicated that the components ; palmitic acid, linoleic acid, Oleic Acid, Pentadecanoic acid, and trans-13-Octadecenoic acid have the biological activities. In agreement with the present results some other researchers, found numerous antimicrobial secondary metabolites, such as gelatinomycin, trichomycin, antibacterial peptides and

chlorotrichomycin are produced by *Trichoderma* spp. **(Maruyama *et al*., 2020).** In addition to acting as antibacterial agents and stimulating plant development, these secondary metabolites provide a wealth of raw materials for the production of antibiotics used in agriculture **(Nawrocka *et al*., 2018).** In addition, pentaibols and other antimicrobial chemicals are produced by most *Trichoderma* strains. These compounds inhibit several plant harmful fungi. Together with enzymes that break down the cell walls of harmful fungi, pentaibols can significantly reduce the growth of these organisms. **(Kovács *et al*., 2021; Martínez *et al*., 2021).** The production of volatile compounds by some *Trichoderma* species is known to hinder colony formation to varying degrees. Evidence suggests that some species of *Trichoderma* can limit colony formation by more than 80%. **(Thambugala *et al*., 2020; Kong *et al*., 2022; Li *et al*., 2022).** Several other researchers reported that the antagonistic effects of *Trichoderma* are attributed to the sequential or simultaneous activity of several pathways **(Alukumbura *et al*., 2022; Chung *et al*., 2022; Sui *et al*., 2022).** According to **Khan *et al*., (2020),** *Trichoderma* spp. create a range of secondary metabolites. These substances include peptaibols, harzianolides, and specific volatile ones. These metabolites have antifungal properties and can boost plant development, making plants more resistant to pathogens.

Conclusion

The present work concluded and confirmed promising results were obtained with using *T. harzianum and T. hamatum* extracts separately or in combination against *F. oxysporum* f.sp. *betae* where the results recorded exhibited strong antifungal activities,So the two *Trichoderma* spp. could be effectively used in the management of sugar beet Fusarium wilt disease. GC/MS analysis of the antifungal materials of both effective *Trichoderma* spp. showed that a numerous compounds produced possessing high antimycotic property.

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الفاعلية المضادة للفطريات لبعض أنواع تريكودرما ضد فيوزاريوم اوكسيبورم أف اسبيشس بيتا المسبب لمرض الذبول لنبات بنجر السكر

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هدفت هذه الدراسة إلى بحث الفاعلية المضادة للفطريات لأربعة أنواع من جنس ترايكودرما(*T. harzianum* ، *T. hamatum* ، *T. viridie* وT. *galaucum*) ضد الفطر الممرض فيوزاريوم اوكسيبورم
والذي يعد من أخطر الفطريات الممرضة التي تنقلها التربة والمسببة لمرض ذبول نبات بنجر السكر. أظهر فحص النشاط المضاد للفطريات باستخدام الطريقتين الثنائية والقرصية أن جميع أنواع الترايكودرما لها تأثير قوى ضد الفيوزاريوم .
وقد سجلت النتائج ان من *T. harzianu* و *mT. hamatum* كانا لهما أعلى تأثير مثبط على النمو للفطر F.o.b.الممرض حيث بلغت نسبة التثبيط ٧٢.٥٣ و٧١.٣٠% و٨٣.٥٠ و٨٠.٠٠% على التوالي .وقد تم دراسة الظروف المثلى لنمو لكلا من نوعي الترايكودرما المختارة الأكثر تثبيطا للفطر الممرض لتحسين إنتاج المواد الضد فطرية على الفطر الممرض وقد أظهرت النتائج ارتفاع كبيرا فى نسبة التثبيط للفطر الممرض F. o. b. وصل الى ٨٥.٥٠ و٨٢.٢٠% على التوالي وتم الحصول على تأثير تثبيطي أكبر وصل الى ٨٦.٧٠% عند استخدام مزيج من راشحي النوعين spp. *Trichoderma* المختارة. وقد تم عمل تعريف جزيئ لكلا من *F. oxysporum f.sp betae* و*T. harzianum* و*T. hamatum* على أساس تسلسل المباعد الداخلي ITS لجينات rDNA. أظهرت النتائج أن *F. oxysporum f.sp betae* أظهر تشابهاً بنسبة ٩٩% مع *F. oxysporum f.sp. betae*، وتم تسجيلها في بنك الجينات برقمPP410242 ، *T. harzianum* أبدى تشابهًا

بنسبة % مع *T. harzianum* وتم تسجيلها فى بنك الجينات برقم PP410286 *وأظهر T. hamatum* تشابهًا بنسبة ١٠٠% مع *T. asperellum* تم تسجيله فى بنك الجينات برقمPP410284

وقد تم تحليل مستخلصات كلا من *T. harzianum* و *T. hamatum* باستخدام تقنية GC/MS لتحديد المكونات الكيميائية الفعالة ضد الفطر الممرض وأظهرت النتائج وجود العديد من المركبات التي تم إنتاجها بواسطة نوعين . *Trichoderma* spp. المختارين لما لهما من خاصية مضادة للفطريات عالية، كانت المكونات هي حمض البالمتيك، وحمض اللينوليك، وحمض الأوليك، وحمض البنتاديكانويك، وحمض ترانس-١٣-أوكتاديسينويك.