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Research Article

Genetic Characterization of *Trichoderma spp.* Isolated from Different Locations of Menoufia, Egypt and Assessment of their Antagonistic Ability

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ABSTRACT

Trichoderma has been used as a biocontrol agent against soil borne diseases that cause economic losses for crops. The objectives of the present investigation were (i) to isolate and characterize *Trichoderma* spp. from Menoufia Governorate and (ii) to evaluate the isolated *Trichoderma* spp. as potential biocontrol agents against some soil borne diseases. Soil samples were collected from nine districts and 25 isolates were obtained. Methods of identification of macroscopic and microscopic features, and the sequences of ITS and TEF1-α yielded three species; *T. harzianum*, *T. longibrachiatum* and *T. asperellum*. Phylogenetic tree of the identified 22 strains confirmed that the two strains *T. longibrachiatum* and *T. asperellum* came together in the same branch while the rest of the strains which were *T. harzianum* were on the other side of the tree. All 25 *Trichoderma* strains and isolates exhibited inhibition to the mycelial growth of four pathogenens. They were antagonized by competition mechanism against *Sclerotium spp.*, by antibiosis against *Fusarium oxysporum* and partially against *Sclerotium spp.* and by mycoparasitism against *Rhizoctonia solani* and *Alternaria alternata*. Also, they elucidated differences in total chitinolytic activity measured by two different methods and exochitonolytic activity. Finally, no correlation was found between total chitinolytic activities and total protein contents.

Keywords: Morphological features; ITS and TEF1-α; Mycoparasitism; Chitinase; Exochitinase

INTRODUCTION

One of the major problems in agricultural production in the world is soil borne diseases that cause significant economic losses in yield and quality of many important crops such as wheat, cotton, vegetables and temperate fruits [1]. The symptoms include root rot, root blackening, wilt, yellowing, stunting or seedling damping-off, bark cracking and twig or branch dieback. They are difficult to predict and control because they form resistant structures that can survive for many years [2]. Currently, many chemical fungicides have been used extensively to control these diseases [3]. These chemicals are expensive, and result in resistant pathogens, environment pollution and bad effect on human health and living organisms. Therefore, biological control is the best alternative method because it is inexpensive, clean and simple. *Trichoderma spp.* have been used as biocontrol agents against these diseases such as *Alternaria alternata*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Scerotium spp.* in many crops [4]. However, precise identification of *Trichoderma* fungi is essential in order to utilize its full application in biocontrol pathogens. Currently, the genus already comprised more than 100 phylogenetically defined species [5]. The taxonomic confirmation of species of the genus *Trichoderma*, based only on morphological markers, can be considered limited and of low accuracy, due to the plasticity of its characteristics [6]. Therefore, molecular techniques must be combined with adopting a variety of parameters in order to identify species correctly [7]. There are several molecular methods to characterize *Trichoderma* species such as internal transcribed spacer rDNA (ITS) and translation elongation factor1-alpha (TEF1-α) [8,9].

Trichoderma spp. biocontrol pathogens by different mechanisms such as antibiotic production, mycoparasitism, production of cell wall degrading enzymes and competition for nutrients or space [10]. However, mycoparasitism has been proposed as the major antagonistic mechanism displayed by *Trichoderma spp.* [11]. *In vitro* dual culture plate technique has been routinely used to select *Trichoderma* strains with high antagonistic activity against pathogens [12]. Cell wall degrading enzymes are the key factors in cell wall destruction is mediated by a set of chitinases, β-(1,4)-, β-(1,3)-

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and β-(1,6)-glucanase, N-acetyl-β-D-glucosaminidase and protease activities and they act synergistically. Therefore, chitinolytic strains (highly producer of chitinase enzymes) of *Trichoderma* are among the most effective agents of biological control of plant diseases [13]. Insoluble substrates such as colloidal chitin coupled to specific dye such as bromocresol purple in agar plate was used as evaluation method for chitinolytic strains [14]. Chitinases can also be assessed sectrophotometrically for N-acetyl-β- D-glucosamine (NAGA) (for total chitinolytic activity) and p-nitrophenol (pNP) (for exochitinase activity) from colloidal chitin supplemented in broth [14]. Therefore, the objectives of the present investigation were (i) to isolate and characterize *Trichoderma spp.* from Menoufia governorate and (ii) to evaluate the isolated *Trichoderma spp.* as potential biocontrol agents against some soil borne diseases.

MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected from the nine districts of Menoufia governorate of Egypt cultivated with different crops Figure 1 and Table 1.

They were collected randomly from the rhizosphere of soils at depths 30-60 cm. They were placed in sterile plastic bags and transferred to laboratory of Genetics Department, Faculty of Agriculture, Menoufia University.

Isolation of *Trichoderma*

Isolation of *Trichoderma spp.* from rhizosphere was made using serial dilution technique [15]. Each composite soil sample was thoroughly mixed and pulverized by means of mortar and pestle, and passed through a 0.5 mm soil screen sieve before 1 g was suspended in 9 ml sterile distilled water. The suspensions were made homogeneous by agitation using a vortex mixer and further serial dilutions of 10-2, 10-3 and 10-4 were made. 0.5 ml aliquot from each dilution was poured in selective media Potato Dextrose Agar (PDA) with 100 µg mL^{-1} streptomycin and plates were then incubated for seven days at 28°C. The culture plates were examined daily and individual green conidia forming *Trichoderma* fungal colonies were isolated [16].

Figure 1: Menoufia governate indicating different districts.

Table 1: *Trichoderma* isolates obtained from districts of Menoufia governorate.

District	No. of location surveyed*	No. of *Trichoderma* isolate
Ashmoun	6	1
El Bagour	14	3
Berket Elsabe	4	1
Tala	15	8
Sadat City	18	2
Shibin El Kom	5	1
El Shohada	7	0
Quwesna	5	1
Menouf	31	8
Total	105	25

*At least five samples were taken from each location

Isolation of *Trichoderma* pure culture

Putative *Trichoderma* colonies were purified by sub culturing on PDA medium. Also, hyphal tipping was performed using a dissecting microscope to view the species of interest at high magnification. In addition, if the spores were small and were difficult to manipulate, another way was used to isolate a pure culture of a particular species by selecting individual spores from the species of interest. Finally, pure cultures were transferred and stored at -4°C for further study.

Morphological characterization of *Trichoderma*

Morphological identification of the potential *Trichoderma* isolates was performed based on macromorphological features including; growth rate and colony characters i.e., color, reverse color and edge and mycelia form and color. Furthermore, micromorphological features including; conidiation, and conidia branching, shape, size and color and also phialides shape, size and disposition were identified.

Radial growth rate (colony diameter) and colony characters were determined on PDA and Synthetic low-Nutrient Agar (SNA), a defined, low-sugar medium [17] media. First, *Trichoderma* isolates were grown on cornmeal dextrose agar (CMD, Difco cornmeal agar 12% (w/v) dextrose) in dark at 20 °C. After seven days 5-mm-diameter plugs were taken from each isolate and inoculated onto freshly prepared PDA or SNA medium. The inoculum plug was placed 1.5 cm from the edge of the Petri dish. They were incubated for seven days in dark at 20, 25, 30 and 35°C. Three replicate plates were tested for each temperature. The colony diameter of each *Trichoderma* isolate was measured every day and the average growth rate per day was calculated. However, all micromorphological data were taken after seven days from colonies grown on CMD containing 100 µg mL^{-1} streptomycin and neomycin at 20–21°C under conditions of 12 h darkness/12 h cool white fluorescent light. Fungal cells from fresh cultures were mounted in water on microscopic slides and examined under a light microscope (SC-CM2000 Biological Trinocular Microscope+LC-21 HD LCD Color Microscope Tablet Camera (5.0 MP) and Viewer, Labmed, Inc., 2728 S. La Cienega Blvd., Los Angeles, CA 90034 USA).

Molecular identification for *Trichoderma* isolates

DNA fungal extraction: The cetyltrimethyl ammonium bromide (CTAB) method was used to extract DNA from isolates. Mycelium

dehydrated in the oven at 45°C for 72 h and the cells were ground finely with a pestle. Genomic DNA was extracted as described by [18] with a few modifications. In brief, the mycelial powder was transferred to an Eppendorf tube and 800 µl lysis buffer (30 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.2 M NaCl, 3% CTAB) supplemented with 0.3% (w/v) β-mercaptoethanol was added. The mixture was incubated at 65 °C for 60 min. An equal volume of chloroforme/isoamyl alcohol [24:1 (v/v)] was added. After homogenization of the mixture and centrifugation at 8000 rpm for 10 min at 4°C, 500 µl supernatant was recuperated into sterile Eppendorf tube. An equal volume of chloroform-isoamylalcohol (24:1) plus 200 µl CTAB 3% (without b-mercaptoethanol) were added and centrifuged again at 8,000 rpm for 10 min. The DNA was precipitated with two volumes of cold 2-propanol supplemented with 10% (w/v) sodium acetate buffer (3 M, pH 8) at -20°C for 2 hrs, washed twice with 500 µl of 70% ethanol, air dried, and resuspended in 200 µl TE buffer (40 mM Tris/HCl pH 8.0, 2 mM EDTA). The final DNA was treated with RNase (4 µl of 10 mg/mL) and incubated at 37°C for 30 min. DNA concentration was estimated by measuring the absorbance at 260 nm. The DNA samples were stored at -20°C for further use.

PCR amplification and DNA sequencing: The ITS and TEF1-α were amplified using primer pairs ITS1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS4 (5'-CCTCCGCTTATTGATATGC-3') [19] and EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and EF1-986R (5'-TACTTGAAGGAACCCTTACC-3') [20], respectively. PCR was performed in a total reaction volume of 50 µl, containing 50 ng of the template DNA, 1.25 U Taq DNA polymerase, 1x Taq polymerase buffer, 0.5 mM of each primer, 200 µM of each of the four deoxyribonucleotide triphosphates. Th PCR amplification of ITS included an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 1 min at 94°C, primer annealing for 2 min at 56.5°C, primer extension for 3 min at 72°C, and a final extension for 5 min at 72°C [21]. As for TEF1-α, the following amplification parameters were used; initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 58.1°C, primer extension for 50s at 74°C, and a final extension for 7 min at 74°C [20]. Finally, amplified products were separated on 1.2% agarose gel in TBE buffer, pre-stained with Ethidium bromide (10 mg/ml) and electrophoresis was carried out at 80 V for 3 h in 1XTBE buffer. PCR products (amplified DNA) were purified and sequenced by Macrogen Inc. (Soul, South Korea).

Sequence submission: Sequences were submitted to GenBank database through Submission Portal (a World Wide Web sequence submission server available at NCBI home page: http://www.ncbi.nlm.nih.gov).

Sequence analysis: The sequences of ITS and TEF1-α of all isolates were analyzed using Molecular Evolutionary Genetics Analysis (MEGA). The sequences were checked and edited manually when needed. The sequenced data were compared against the Gene Bank database (http://www.ncbi.nlm.nih.gov/BLAST/), where a nucleotide blast program was chosen to identify the homology between the PCR fragments and the sequences on the Gene Bank database. Besides, the ITS sequences were compared to a specific database for *Trichoderma* using *Trich*OKEY 2 program, which available online from the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (ISTH, www.isth.info) [22]. Finally, the cross ponding species for each isolate was identified and a specific laboratory nomenclature system for identified strains was followed.

Phylogenetic analysis of the sequence data

ITS DNA sequences were aligned using the multiple sequence alignment program Clustal-X [23], and then visually adjusted. Single gaps were treated as missing data. Phylogenetic analyses were performed in MEGA5 [24]. A parsimony analysis was performed using a heuristic search, with a starting tree obtained via stepwise addition, with random addition of sequences with 1000 replicates. Stability of tree was assessed with 1000 bootstrap replications. Out-group taxa for the individual analysis were determined from a preliminary, broad based phylogenetic analysis of *Trichoderma* sections and the tree.

Dual-culture antagonistic activity assay

The antagonistic effect of each strain or isolate was tested using dual-culture technique against four pathogens namely; *Alternaria alternate*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Scerotium spp.* [25]. The tested *Trichoderma* isolates were grown on PDA medium at 20°C for 6-days. Disks of 5-mm diameter from each isolate were inoculated on PDA medium in one side in Petri dish and the opposite side was inoculated by pathogen disk. Plate with pathogen only was used as control. After incubation of five and seven days periods at 25 ± 2°C with alternate light and darkness, data on growth zones and colony diameters were recorded. Percent growth inhibition against growth of pathogen was calculated by the formula:

%Inhibition of Radial Growth (PIRG)=(R1 - R2)/R1*100 where,

R1, radius of pathogen mycelium in the control plate

R2, radius of pathogen mycelium in the dual culture plate (*Trichoderma* and pathogen).

Also, classification of strains and isolates for antagonistic to fungal plant pathogens was done after seven days of growth based on [26,27] with some modifications as follows:

Class 1 Biocontrol agent grew and stopped without contact with the colony of pathogen and a zone of growth inhibition exists between the fungi.

Class 2 Biocontrol agent grew and stopped without contact with the colony of pathogen

Class 3 Biocontrol agent grew and overlapped the colony of pathogen

Chitinase activity

Preparation of colloidal chitin: Colloidal chitin was prepared according to the modified method described by [28]. Ten grams of practical grade crab shell chitin were mixed with 150 ml 10 N HCl with continuous stirring at 4°C for overnight. The suspension was repeatedly mixed with one-litre water and filtered through a course filter paper. This step was repeated four to five times and the pH of the suspension was adjusted to 7.0 by addition of 5 N NaOH and the colloidal suspension was washed several times with distilled H_2O for desalting. After desalting, the suspension was centrifuged at 5000 rpm for 10 min and the precipitate was collected for further use as colloidal chitin.

Aagar medium zone measurement: Chitinase detection medium consisted of a basal medium comprising (per liter) 0.3 g of $MgSO_4.7H_2O$, 3.0 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, 1.0 g of

citric acid monohydrate, 15 g of agar, 200 µl of Tween-80, 4.5 g of colloidal chitin and 0.15 g of bromocresol purple; pH was adjusted to 4.7 and then autoclaved at 121°C for 15 min. Fresh culture plugs of the isolates to be tested for chitinase activity were inoculated into the medium and incubated at 25±2°C and were observed for colored zones formation and diameters were measured after 72 h [14].

Spectrophotometric determination: Culture plugs containing young actively growing mycelium of *Trichoderma* isolates were inoculated in colloidal chitin supplemented broth and incubated at 28°C for five days at 200 rpm. Cultural filtrates obtained by filtering through Whatman No. 1 filter paper were stored at -20°C until further use. Filtrates were analyzed through spectrophotometric assay for N-acetylglucosamines and total chitinolytic activities was detected as described by [14].

Total chitinolytic activity: Total chitinolytic activity was assayed by measuring the release of reducing saccharides from colloidal chitin. A reaction mixture containing 1 ml of culture supernatant, 0.3 ml of 1 M sodium acetate buffer (SA-buffer), pH 4.6 and 0.2 ml of colloidal chitin was incubated at 40°C for 20 h and then centrifuged at 13,000 rpm for 5 min at 6°C. After centrifugation, an aliquot of 0.75 ml of the supernatant, 0.25 ml of 1% solution of dinitrosalycilic acid in 0.7 M NaOH and 0.1 ml of 10 M NaOH were mixed in 1.5 ml micro centrifuge tubes and heated at 100°C for 5 min. Absorbance of the reaction mixture at A582 was measured after cooling to room temperature against the blank [29]. Calibration curve with N-acetyl-β–D-glucosamine (NAGA) was used as a standard to determine reducing saccharide concentration. Under the assay conditions described, a linear correlation between A582 and NAGA concentration was found in the interval of 40-800 mg/ml NAGA. Chitinolytic activity was estimated in terms of the concentration (mg/ml) of NAGA released. One unit of chitinase is defined as the release amount of enzyme which releases micromole of NAGA per hour under the reaction condition [30].

Exochitinase activity: N-acetyl-β-D-glucosaminidase (exochitinase) activity was measured and monitored spectrophotometrically as the release of p-nitrophenol (pNP) from p-nitrophenyl-Nacetyl-β-D-glucosaminide (pNPg) A mixture of 25 µl of culture filtrate, 0.2 ml of pNPg solution (1 mg pNPg ml^{-1}), and 1 ml of 0.1 M of sodium acetate buffer (pH 4.6) was incubated at 40°C for 20 h and then centrifuged at 13,000 rpm. An aliquot of 0.3 ml of 0.125 M Sodium tetraborate–NaOH buffer (pH 10.7) was added to 0.6 ml of supernatant, absorbance at 400 nm (A400) was measured immediately after mixing and pNP concentration (in terms of Volume Activity) in the solution was calculated using the pNP molar extinction coefficient (18.5 mM^{-1} - cm^{-1}) with the help of following formula:

Volume Activity U/ml=[Δ OD (OD test-OD blank) x Vt x df]/ (18.5 x t x 1.0 x Vs)

Where,

Vt=Total volume (900 µl); Vs=Sample volume (25 µl); 18.5=Millimolar extinction coefficient of p-nitrophenol under the assay condition (cm^2/micromole); 1.0=Light path length (cm); t=Reaction time (20 hours=1200 minutes); df=Dilution factor [29].

One unit (U) of exo-chitinase activity is defined as the amount of enzyme that is required to release 1 mol of N-acetyl-b-Dglucosamine per minute from 0.5% of dry colloidal chitin solution under standard bioassay conditions and expressed as Units per gram dry substrate (U gds^{-1}) [31].

Protein estimation

The protein content in the culture filtrates was estimated by the dye-binding method of [32]. The amount of protein was calculated using Bovine Serum Albumin (Sigma Co.) as standard.

Data analysis

Analyses of the mean values for percent inhibition of radial growth of different pathogenic fungi and diameter of the colored zone on agar medium were carried out with ANOVA. The differences were calculated with Duncan's multiple range test ($P<0.05$) using statistical package SPSS Version 25.0.

RESULTS

In the present investigation a *Trichoderma* survey was conducted from different locations of Menoufia governate. The total number of locations surveyed was 105. Summarized list of total number of locations surveyed and the 25 obtained *Trichoderma* isolates has been provided in Table 1.

Colony characteristics of different isolates of *Trichoderma* have been given in Table 2.

Colony growth rate per day of different isolates at different temperatures has been provided in Table 3.

Based on observations recorded there were presence of noticeable differences in their growth rate and in their time for full colonization (Figure 2).

Also, the microscopic features including conidia and phialides characters of *Trichoderma* isolates were observed under light microscope (Figure 3).

Isolates varied in their microscopic features markedly (Table 4).

The results of morphological identification were used to identify the species of *Trichoderma* isolates according to the method of [33,34].

ITS and TEF1-α sequences were submitted to NCBI Genbank and accession numbers were given as shown in Table 5. The BLAST and *Trich*OKEY search results were presented in Table 5. Of the 25 *Trichoderma* isolates, 22 were identified at the species level by analysis of their ITS sequences. Also, 10 isolates were confirmed by TEF1-α sequences analysis. According to the analyses, 20 isolates were identified as *T. harzianum*, one isolate was *T. longibrachiatum* and another one was *T. asperellum*. Corresponding laboratory strain code was given to 22 identified strains (Table 5). Phylogenetic tree was constructed to graphically represent the genetic relationship of the 22 strains (Figure 4).

Trichoderma strains and isolates were evaluated for their ability to antagonize four economically important plant pathogens namely; *Alternaria alternata*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium spp.* using dual culture method. All *Trichoderma* strains and isolates exhibited inhibition to the mycelial growth of all pathogens after five and seven days (Table 6 and Figure 5).

The lowest inhibition percentage was 24.96% by MNF-MAS-Tricho6 after five days with *Sclerotium spp.*, and the highest was 100% by MNF-MAS-Tricho5 with *Sclerotium spp.* Also, the same *Trichoderma* strain or isolate showed big difference in their inhibition percentage with different pathogens. At the same time, significant differences were noticed among *Trichoderma* strains and isolates in their inhibition percentages of the same pathogen.

Table 2: Colony and mycelial characters of different *Trichoderma* isolates cultured on PDA after seven days of incubation in dark at 28°C.

Strain	Colony			Mycelial	
	Color [a]	Reverse color [b]	Edge	Form	Color
Tricho1	Dark green	Colorless	Wavy	Ring like zone	Watery white
Tricho2	White to green	Creamish	Wavy	Concentric zones	White
Tricho3	Yellow to green	Creamish	Smooth	Concentric zones	White
Tricho4	Dark green	Light yellow	Smooth	Ring like zone	White
Tricho5	Dark green	Colorless	Wavy	Ring like zone	White
Tricho6	Dark green	Colorless	Smooth	Concentric zones	White
Tricho7	White to green	Light yellow	Smooth	Concentric zones	White
Tricho8	Yellowish green	Colorless	Smooth	Concentric zones	Watery whit
Tricho9	Dark green	Light yellow	Smooth	Concentric zones	White
Tricho10	Dark green	Colorless	Smooth	Ring like zone	White
Tricho11	Dark green	Colorless	Smooth	Concentric zones	Watery whit
Tricho12	Yellowish green	Colorless	Smooth	Concentric zones	Watery whit
Tricho13	Dark green	Creamish	Smooth	Ring like zone	White
Tricho14	Dark green	Creamish	Smooth	Ring like zone	White
Tricho15	Yellowish green	Creamish	Wavy	Concentric zones	White
Tricho16	White to green	Colorless	Smooth	Ring like zone	Watery whit
Tricho17	Yellowish green	Light yellow	Smooth	Ring like zone	White
Tricho18	Dark green	Creamish	Smooth	Ring like zone	White
Tricho19	Dark green	Colorless	Smooth	Concentric zones	White
Tricho20	Yellowish green	Light yellow	Smooth	Concentric zones	Watery whit
Tricho21	Dark green	Colorless	Smooth	Concentric zones	Watery whit
Tricho22	Dark green	Colorless	Smooth	Ring like zone	Watery whit
Tricho23	Dark green	Colorless	Smooth	Concentric zones	White
Tricho24	Dark green	Colorless	Smooth	Ring like zone	White
Tricho25	Dark green	Colorless	Smooth	Concentric zones	White

[a] colony color from the front side growth of colony, [b] colony color from the back-side view

Table 3: *Trichoderma* isolate average growth rate per day cultured on PDA and SNA in dark for seven days at different temperatures.

Isolate code	Colony diameter/day (cm)							
	PDA				SNA			
	20 °C	25 °C	30 °C	35 °C	20 °C	25 °C	30 °C	35 °C
Tricho1	1.91	2.49**	2.85**	0.83	1.64	2.3***	2.17***	0.7
Tricho2	1.65	2.41**	2.69**	0.6	1.45	2.45***	2.34***	1.3
Tricho3	1.76 ***	2.45**	2.49**	0.83	1.61	2.11***	1.96***	0.65
Tricho4	2.28	3.41**	6.2*	6.14*	1.3	2.26***	2.32***	1.47
Tricho5	2.01	2.73**	2.83**	0.93	1.67	2.52**	1.71**	0.86
Tricho6	1.42	2.57**	2.44**	0.79	1.56	2.42***	2.14***	1.1
Tricho7	1.48	2.13**	2.41	1.31	1.11	1.85***	1.97***	1.14
Tricho8	1.72	2.68**	2.61**	0.79	1.63	2.36**	2.54***	1.13
Tricho9	1.94	2.62**	2.56**	0.62	1.34	2.16***	1.94***	0.48
Tricho10	1.64	2.61**	2.68*	0.86	1.58	2.25**	2.14***	0.71
Tricho11	1.71 ***	2.35**	2.3**	0.95	1.36	2.41**	1.96***	1.11
Tricho12	1.87 ***	2.71**	2.88	1.24	1.68	2.27**	1.95***	0.5
Tricho13	1.81 ***	2.58**	2.86**	0.83	1.61	2.4***	2.18***	1.2
Tricho14	1.95	2.65**	2.85*	1.79	1.54	2.2**	2.09***	1.11
Tricho15	1.91	2.83**	2.82**	0.8	1.59	2.52***	2.1***	0.9
Tricho16	2.39 ***	3.01**	2.83**	0.86	1.4	2***	1.92***	0.75
Tricho17	2.3 ***	2.97**	3.2**	1.17	1.83***	2.5***	2.23***	0.95
Tricho18	1.92 ***	2.92**	3.32*	0.88	1.78***	2.55**	2.29***	0.48
Tricho19	2.21	2.53*	2.72**	0.88	1.43	2.23***	2.09***	0.8

Tricho20	1.39	2.54**	2.7**	0.75	1.38	2.02**	1.58***	0.4
Tricho21	1.78	2.36**	2.19**	0.65	1.24	2.37***	1.99***	0.78
Tricho22	1.85 ***	2.4**	2.59***	0.54	1.54	2.22**	2.22***	0.77
Tricho23	1.63	2.32**	2.34**	0.72	1.44	2.27***	2.22***	0.87
Tricho24	1.58	2.56**	2.23***	0.86	1.32	2.2**	1.97***	0.97
Tricho25	1.77	2.22**	1.94***	0.39	1.4	2.36**	2.1***	0.73

*fully cover colonialized after 2 days.
** fully cover colonialized after 3days.
*** fully cover colonialized after 4 days

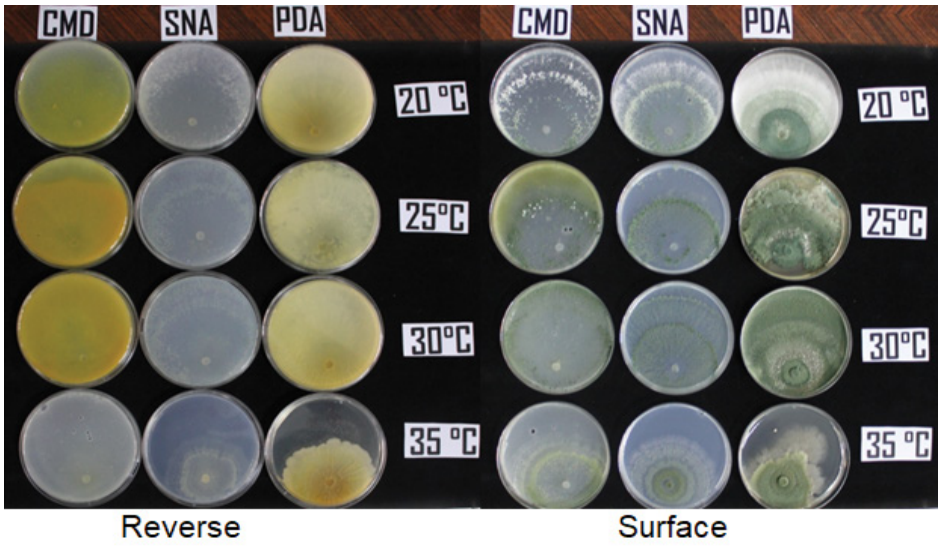

Figure 2: Surface and reverse of morphological growth of *Trichoderma harzianum* colonies on three different cultures after seven days at different temperatures.

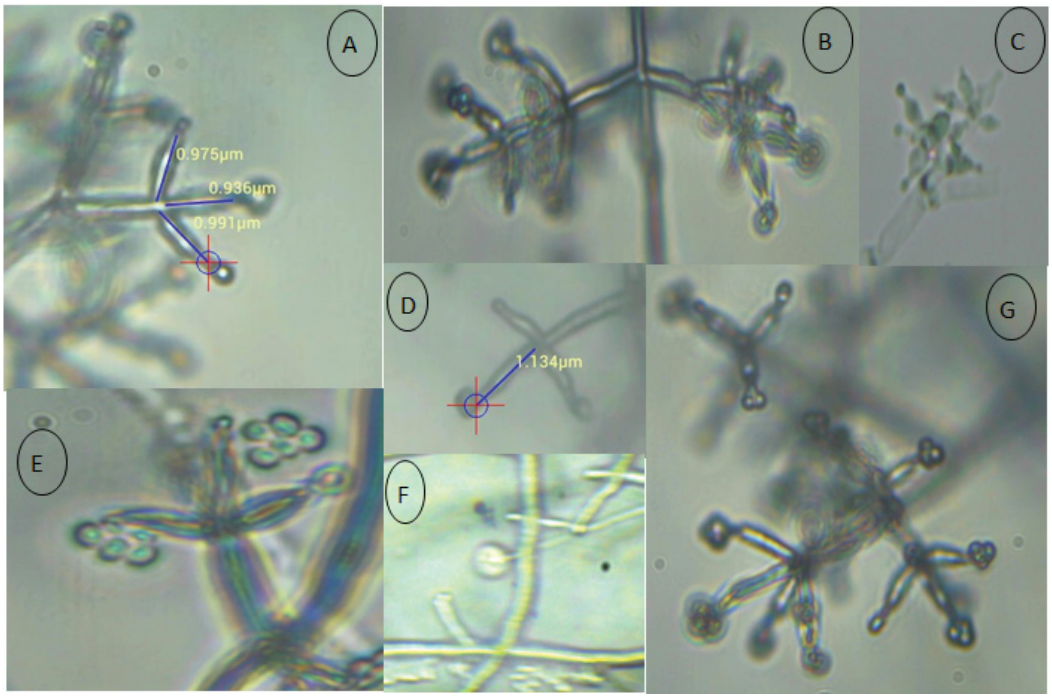

Figure 3: Isolates microscopic features (A) Phialides size of *Trichoderma harzianum* (B) Phialides disposition (C) Phialides of *Trichoderma longibrachiatum* (D) Phialides of *Trichoderma asperellum* (E) Conid

For *Alternaria alternate*, the highest inhibition for its growth was 75.97% with MNF-MAS-Tricho5 after five days and the lowest was 49.30% with MNF-MAS-Tricho4 and after seven days the same strains had the highest and lowest inhibition with values 78.52% and 61.97%, respectively. In the case of *Fusarium oxysporum*, the highest inhibition was by the strain MNF-MAS-Tricho22 after five and seven days (70.05% and 77.97%, respectively) and the lowest inhibition was by MNF-MAS-Tricho11 after five and seven days (45.16% and 59.66%, respectively). As for *Rhizoctonia solani*, the highest inhibition was by MNF-MAS-Tricho2 with values 71.77%

Table 4: Conidia and phialides characters of *Trichoderma* isolates cultured on CMD at 20–21 °C after seven days of incubation under 12 h darkness/12 h conditions.

Isolate code	Conidia					Phialides		
	Conidiation	Branching	Shape	Size (µm)	Colour	Shape	Size (µm)	Disposition
Tricho1	Concentric zones	Branched	ellipsoidal, sub globose	1.5-3.4	Green	Nine- Pin shape	6-14 x1.4-2.6	Clustered (2-3)
Tricho2	Concentric zones	Highly branched	ellipsoidal, globose	1.3- 3.6	Green	Nine- Pin shape	7-15 x2-3	Clustered (2-3)
Tricho3	Concentric Zones	Branched	sub globose	1.4- 3.8	Green	Pin shape	4.8-11.4 ×2.1-2.8	Solitary
Tricho4	Ring like zones	Highly branched	ellipsoidal, sub globose	1.7-4.1	Green	Nine-Pin shape	6-14 x1.4-3	Solitary
Tricho5	Concentric zones	Highly branched	ellipsoidal, sub globose	1.4-3.7	Green	Nine-Pin shape	5.6-14.8 x 2-3	Clustered (2-3)
Tricho6	Ring like zones	Branched	ellipsoidal, globose	1.3-3.3	Green	Globose	4.9-11.2 × 1.9-3	Clustered (2-3)
Tricho7	Concentric Zones	Highly branched	ellipsoidal	1.4-3.9	Dark green	Globose	6.5- 11.5× 2.6-3.5	Clustered (2-3)
Tricho8	Concentric zones	Highly branched	ellipsoidal, sub globose	1.5- 3.4	Light Green	Nine- Pin shape	5.9-15.2 × 1.9-2.8	Solitary
Tricho9	Concentric zones	Moderately branched	ellipsoidal, globose	1.5- 3.6	Green	Nine- Pin shape	7-14.8×1.9-2.6	Clustered (2-3)
Tricho10	Ring like zones	Highly branched	ellipsoidal, globose	1.4- 3.8	Green	Nine-Pin shape	6.2-10.2×2.2-2.9	Clustered (2-3)
Tricho11	Concentric zones	Highly branched	ellipsoidal, globose	1.5- 3.9	Green	Nine-Pin shape	5.8-12.4×2.7-3.2	Clustered (2-3)
Tricho12	Concentric zones	Highly branched	ellipsoidal, obovoid	1.4-3.9	Dark Green	Nine-Pin shape	6.5-11.7×2.7-3.5	Clustered (2-3)
Tricho13	Ring like zones	Moderately y branched	ellipsoidal, obovoid	1.5-3.8	Light Green	Nine- Pin shape	6.1-12.5×2.7-3	Clustered (2-3)
Tricho14	Concentric zones	Highly branched	ellipsoidal, obovoid	1.4-3.6	Light Green	Nine- Pin shape	5.6-15x1.4-3	Clustered (2-3)
Tricho15	Concentric zones	Highly branched	ellipsoidal, obovoid	1.3-3.6	Green	Nine-Pin shape	6.8-14.4 × 2.2-3.2	Clustered (2-3)
Tricho16	Concentric zones	Branched	ellipsoidal, obovoid	1.4-3.5	Green	Nine-Pin shape	5.5-13.7 × 1.7-3.2	Clustered (2-3)
Tricho17	Ring like zones	Branched	ellipsoidal, obovoid	1.5- 3.9	Light Green	Nine-Pin shape	4.5-12.0 × 1.7-3.0	Clustered (2-3)
Tricho18	Concentric zones	Moderately branched	ellipsoidal, sub globose	1.3-3.9	Dark Green	Nine-Pin shape	3.9-13.7× 1.7-2.9	Clustered (2-3)
Tricho19	Concentric zones	Branched	ellipsoidal, sub globose	1.6-3.0	Light Green	Globose	8-14 x2-3	Clustered (2-3)
Tricho20	Ring like zones	Highly branched	ellipsoidal, sub globose	1.7-4.1	Green	Nine-Pin shape	6-14 x1.4-3	Solitary
Tricho21	Concentric zones	Highly branched	ellipsoidal, sub globose	1.4-3.7	Green	Nine-Pin shape	5.6-14.8 x 2-3	Clustered (2-3)
Tricho22	Ring like zones	Branched	ellipsoidal, globose	1.3-3.3	Green	Globose	4.9-11.2 × 1.9-3	Clustered (2-3)
Tricho23	Concentric zones	Branched	ellipsoidal, globose	1.5-3.4	Light Green	Nine- Pin shape	6-15 x1.4-2.8	Solitary
Tricho24	Concentric zones	Highly branched	ellipsoidal, sub globose	1.5-3.4	Light Green	Nine- Pin shape	5.9-15.2 × 1.9-2.8	Solitary
Tricho25	Concentric zones	Moderately branched	ellipsoidal, globose	1.5-3.6	Green	Nine- Pin shape	7-14.8×1.9-2.6	Clustered (2-3)

Table 5: Molecular identification of *Trichoderma* isolates by ITS region and *tef*1gene nucleotide sequences.

Isolate code	Species identification	NCBI GenBank accession number		Corresponding Strain code	Origin
		ITS	*tef*1		
Tricho1	*Trichoderma harzianum*	MH688753	MK295193	MNF-MAS-Tricho1	Quesna
Tricho2	*Hypocrea lixii/Trichoderma harzianum*	MH688857	————	MNF-MAS-Tricho2	El-Bagour
Tricho3	*Trichoderma longibrachiatum*	MH707326	————	MNF-MAS-Tricho3	Sadat city
Tricho4	*Trichoderma harzianum*	MH712434	MK295194	MNF-MAS-Tricho4	Menouf
Tricho5	*Hypocrea lixii/Trichoderma harzianum*	MH697665	MK295195	MNF-MAS-Tricho5	Ashmoun
Tricho6	*Trichoderma harzianum*	MH699073	————	MNF-MAS-Tricho6	Shibin El Kom
Tricho7	*Trichoderma asperellum*	MH688914	————	MNF-MAS-Tricho7	Menouf
Tricho8	*Hypocrea lixii/Trichoderma harzianum*	MH697403	————	MNF-MAS-Tricho8	El-Bagour
Tricho9	*Trichoderma harzianum*	MH702379	MK295196	MNF-MAS-Tricho9	El-Bagour
Tricho10	*Trichoderma harzianum*	MH697404	————	MNF-MAS-Tricho10	Menouf
Tricho11	*Hypocrea lixii/Trichoderma harzianum*	MH697405	————	MNF-MAS-Tricho11	Sadat city
Tricho12	*Hypocrea lixii/Trichoderma harzianum*	MH697536	MK295197	MNF-MAS-Tricho12	Birket El Sab
Tricho13	*Hypocrea lixii/Trichoderma harzianum*	MH697554	MK295198	MNF-MAS-Tricho13	Menouf
Tricho14	*Hypocrea lixii/Trichoderma harzianum*	MH697555	————	MNF-MAS-Tricho14	Tala
Tricho15	*Trichoderma sp.*	————	————	Tricho15	Tala
Tricho16	*Hypocrea lixii/Trichoderma harzianum*	MH697561	————	MNF-MAS-Tricho16	Tala
Tricho17	*Trichoderma harzianum*	MH697573	MK295199	MNF-MAS-Tricho17	Tala
Tricho18	*Hypocrea lixii/Trichoderma harzianum*	MH697572	————	MNF-MAS-Tricho18	Tala
Tricho19	*Hypocrea lixii/Trichoderma harzianum*	MH697574	MK295200	MNF-MAS-Tricho19	Tala
Tricho20	*Trichoderma harzianum*	MH697684	MK295201	MNF-MAS-Tricho20	Tala
Tricho21	*Trichoderma sp.*	————	————	Tricho21	Tala
Tricho22	*Hypocrea lixii/Trichoderma harzianum*	MH697598	————	MNF-MAS-Tricho22	Menouf
Tricho23	*Hypocrea lixii/Trichoderma harzianum*	MH697609	————	MNF-MAS-Tricho23	Menouf
Tricho24	*Trichoderma harzianum*	MH703537	MK295202	MNF-MAS-Tricho24	Menouf
Tricho25	*Trichoderma sp.*	————	————	Tricho25	Menouf

and 79.67% after five and seven days, respectively, however the lowest was by MNF-MAS-Tricho11 with values 43.71% and 60.02% after five and seven days, respectively. Finally, *Sclerotium spp.* the highest inhibition was by MNF-MAS-Tricho5 after five and seven days (100%) while the lowest was by MNF-MAS-Tricho3 after five and seven days (24.96% and 34.72%, respectively).

In addition, the antagonistic ability of *Trichoderma* was classified into three classes. These were antibiosis class namely Class I, competition class namely Class 2 and mycoparasitism class namely Class 3 (Table 7 and Figure 6).

For *Alternaria alternate*, all strains and isolates showed Class 3 antagonistic ability after five days except three of them. However, after seven days all of them showed mycoparasitism ability of antagonistic ability. As for *Fusarium oxysporum*, 20 *Trichoderma spp.* showed antibiosis Class1 type of antagonism after five days, while at the end of seven days 16 indicated mycoparasitism Class3 antagonism. Eight of *Trichoderma spp.* demonstrated antibiosis Class1 antagonism by the end of the seventh day. The three strains; MNF-MAS-Tricho1, MNF-MAS-Tricho9 and MNF-MAS-Tricho12 continuous to have antibiosis Class1 reaction at five and seven days.

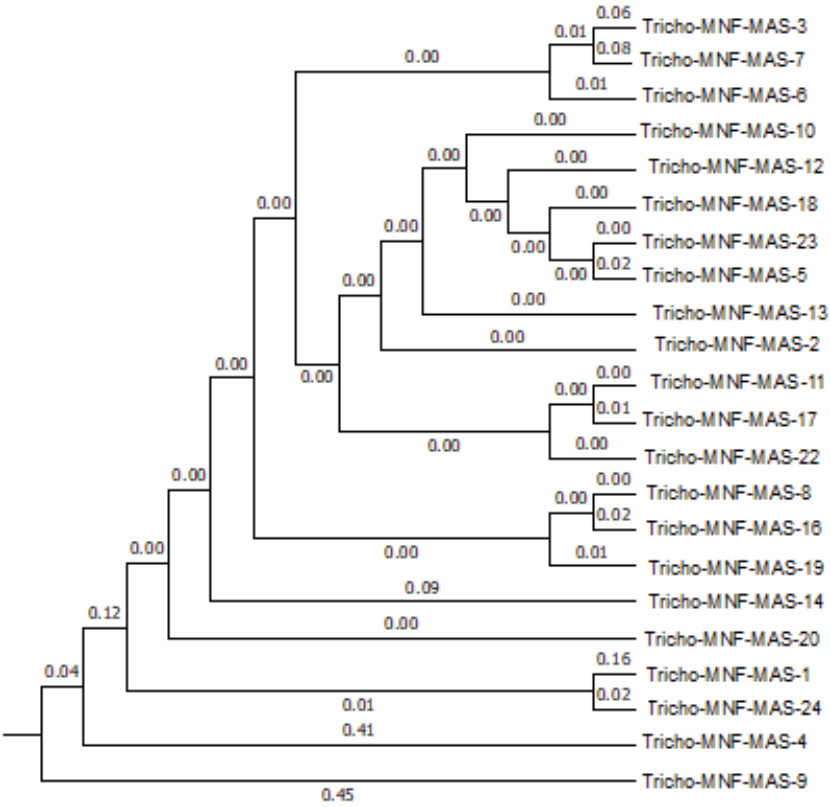

Figure 4: Neighbor-joining phylogenetic tree of 22 *Trichoderma* strains using Maximum Parsimony based on internal transcribed spacer rDNA (ITS) sequences by MEGA5 program. Numbers indicate genetic relationship among strains.

Only one strain namely; MNF-MAS-Tricho16 had compotation Class2 type of reaction for all times. In the case of *Rhizoctonia solani*, *Trichoderma* demonstrated only Class3 type of antagonism for all times. Finally, *Sclerotium spp.* fungi, 12 *Trichoderma* indicated Class1 antagonism, seven Class2 and six Class3 after five days. Same results were obtained after seven days except three of Class1 showed Class3 antagonism.

Finally, for quantifying the chitinase activity and find out the differences among strains in their chitinase activities many experiments were conducted. Results were presented in Table 8.

At the beginning *Trichoderma* strains and isolated were inoculated on chitinase media containing colloidal chitin and bromocresol purple (pH indicator). Breaking down of chitin into N-acetyle glucoseamine by *Trichoderma* causes change in the pH from alkaline to acidic which indicated by changing in the color of the media from yellow forming a purple zone surrounding the inoculate. *Trichoderma* strains and isolates showed significant differences in the diameters of formed zones. However, MNF-MAS-Tricho10, MNF-MAS-Tricho12, MNF-MAS-Tricho22, MNF-MAS-Tricho24 and isolate Tricho25 did not form colored zone at all. The highest value was 83.7 for MNF-MAS-Tricho20 and the lowest value was 60.03 for Tricho21. Also, total chitinolytic activity was determined spectrophotometrically by measuring the release of reducing saccharides from colloidal chitin in culture filtrate of all isolates and strains. Differences in enzyme activities among isolates and strains were noticed. The highest value was 7.78 for U/ml for Tricho15 and the lowest was 1.04 U/ml for Tricho25. However, the five strains and isolates MNF-MAS-Tricho10, MNF-MAS-Tricho14, MNF-MAS-Tricho22, MNF-MAS-Tricho24 and isolate Tricho25 demonstrated enzyme activity with values; 3.93, 7.76, 2.22, 3.48 and 1.04, respectively. Although, these five strains and isolates did not show any colored zone in the first test. At the same time, another enzyme activity was measured for all strains and isolates namely; exochitinase. It was measured as release of p-nitrophenol from p-nitrophenyl-N-acetyl-β-D-glucosaminide in culture filtrates. The isolates and strains expressed differential exochitinase activity. The highest value was 0.0256 for MNF-MAS-Tricho12 and the lowest was 0.0005 for Tricho25. Protein content was recorded in culture filtrates of all stains and isolates. The highest amount of protein (315 μg ml^{-1}) was estimated in the strain MNF-MAS-Tricho14. On the other hand, the lowest protein content of 25 μg/ml was recorded in the culture filtrates of the MNF-MAS-Tricho23 (Table 8).

DISCUSSION

Species of the important biocontrol agent *Trichoderma* are distributed worldwide and each one has its own ecological preference [35]. In this study, 25 isolates of *Trichoderma* have been characterized. The isolates represented 105 locations (about 525 soil samples), collected from different cultivated field with different crops from the nine districts of Menoufia governorate. The isolates showed the formation of concentric rings that are typical of *Trichoderma spp.* colonies, where the green color of the conidia is interleaved with the white of the mycelium, which is consistent with the characteristics previously described for this fungus [5,36]. However, isolates exhibited variability in macroscopic features such as colony and mycelial characters, and colony growth rate and also, in their microscopic features such as conidia and phialides characters. However, although the colony

Table 6: Percent inhibition in mycelial growth of different pathogenic fungi by different strains of *Trichoderma* after 5 and 7 days incubation period in the dual culture plate.

Strain/isolate	Percent Inhibition of Radial Growth							
	Alternaria alternate		*Fusarium oxysporum*		*Rhizoctonia solani*		*Sclerotium spp.*	
	After 5 days	After 7 days	After 5 days	After 7 days	After 5 days	After 7 days	After 5 days	After 7 days
MNF-MAS-Tricho1	69.64 ^{def}	75.35 ^{cdefg}	55.45 ^{bcde}	67.23 ^{bcde}	71.66 ^{de}	76.99 ^{de}	64.17 ^{defg}	68.83 ^{efgh}
MNF-MAS-Tricho2	59.78 ^{abcde}	68.78 ^{abcdef}	62.21 ^{cdefg}	72.20 ^{cdefg}	71.7^{7}e	79.6^{7}e	48.02^{bc}	54.78 ^{bcd}
MNF-MAS-Tricho3	59.15 ^{abcd}	69.37 ^{abcdef}	53.30 ^{abc}	65.65 ^{abc}	55.02^{b}	68.06^{b}	36.67^{b}	44.91^{ab}
MNF-MAS-Tricho4	49.30 a	61.9^{7}a	60.37 ^{bcdef}	70.85 ^{bcdef}	60.25 ^{bcde}	71.77 ^{bcde}	38.80^{b}	46.76^{bc}
MNF-MAS-Tricho5`	75.9^{7} f	78.5^{2} ^{f}g	55.30 ^{bcde}	67.12 ^{bcde}	66.16 ^{bcde}	75.97 ^{bcde}	100.00^{k}	100.00^{k}
MNF-MAS-Tricho6	55.24 ^{abcd}	66.43 ^{abcde}	54.99 ^{bcd}	66.89 ^{bcd}	65.31 ^{bcde}	75.36 ^{bcde}	24.9^{6}a	34.7^{2}a
MNF-MAS-Tricho7	73.55 ^{ef}	76.99^{g}	54.53 ^{bcd}	66.55 ^{bcd}	67.60 ^{cde}	76.99 ^{de}	57.76 ^{cdef}	63.26 ^{defg}
MNF-MAS-Tricho8	57.43 ^{abcd}	68.08 ^{abcdef}	54.69 ^{bcd}	66.67 ^{bcd}	62.33 ^{bcde}	73.25 ^{bcde}	68.08 ^{efg}	68.83 ^{efgh}
MNF-MAS-Tricho9	69.33 ^{def}	77.00 ^{defg}	51.61 ^{ab}	64.41 ^{ab}	62.59 ^{bcde}	73.43 ^{bcde}	51.53^{cd}	57.84 ^{cde}
MNF-MAS-Tricho10	65.41 ^{bcdef}	74.06 ^{bcdefg}	53.00 ^{abc}	65.42 ^{abc}	61.14 ^{bcde}	72.40 ^{bcde}	61.86 ^{defg}	66.82 ^{defgh}
MNF-MAS-Tricho11	52.39 ^{ab}	64.30 ^{ab}	45.16 a	59.6^{6}a	43.7^{1}a	60.0^{2}a	81.55 ^{hij}	83.95 ^{ij}
MNF-MAS-Tricho12	60.63 ^{abcde}	70.47 ^{abcdefg}	68.20 ^{fg}	76.61 ^{fg}	60.37 ^{bcde}	71.86 ^{bcde}	70.91 ^{fghi}	74.69 ^{ghij}
MNF-MAS-Tricho13	65.23 ^{bcdef}	73.92 ^{bcdefg}	64.82 ^{efg}	74.12 ^{efg}	59.86 ^{bcde}	71.50 ^{bcde}	57.07 ^{cde}	62.65 ^{defg}
MNF-MAS-Tricho14	53.83 ^{abc}	65.38 ^{abc}	56.68 ^{bcde}	68.14 ^{bcde}	59.35 ^{bc}	71.14 ^{bcde}	60.62 ^{cdef}	65.74 ^{defgh}
Tricho15	55.56 ^{abcd}	66.67 ^{abcde}	58.99 ^{bcdef}	69.83 ^{bcdef}	58.08 ^{bc}	70.23 ^{bcd}	84.03^{j}	86.11^{j}
MNF-MAS-Tricho16	63.85 ^{bcdef}	72.89 ^{bcdefg}	60.83 ^{bcdefg}	71.19 ^{bcdefg}	65.05 ^{bcde}	75.18 ^{bcde}	74.51 ^{ghij}	77.82 ^{hij}
MNF-MAS-Tricho17	69.64 ^{def}	77.23 ^{efg}	61.60 ^{cdefg}	71.75 ^{cdefg}	61.65 ^{bcde}	72.77 ^{bcde}	62.04 ^{defg}	66.98 ^{defgh}
MNF-MAS-Tricho18	67.61 ^{cdef}	75.70 ^{cdefg}	60.52 ^{bcdef}	70.96 ^{bcdef}	61.14 ^{bcde}	72.40 ^{bcde}	55.47 ^{cde}	61.27 ^{def}
MNF-MAS-Tricho19	51.64 ^{ab}	63.73^{ab}	52.84 ^{abc}	65.31 ^{abc}	59.44 ^{bcd}	71.20 ^{bcde}	64.52 ^{defg}	69.14 ^{efgh}
MNF-MAS-Tricho20	57.59 ^{abcd}	68.19 ^{abcdef}	64.36 ^{defg}	73.79 ^{defg}	66.84 ^{bcde}	76.45 ^{bcde}	74.45 ^{ghi}j	77.78 ^{hij}
Tricho21	67.14 ^{cdef}	75.35 ^{cdefg}	52.84 ^{abc}	65.31 ^{abc}	56.29 ^{bc}	68.96 ^{bc}	55.30 ^{cde}	61.11 ^{def}
MNF-MAS-Tricho22	61.66 ^{abcde}	71.24 ^{abcdefg}	70.0^{5}g	77.9^{7}g	63.78 ^{bcde}	74.28 ^{bcde}	68.96 ^{efgh}	72.99 ^{fghi}
MNF-MAS-Tricho23	55.87 ^{abcd}	66.90 ^{abcde}	55.76 ^{bcde}	67.46 ^{bcde}	56.17^{bc}	68.88^{bc}	53.52 ^{cd}	59.57^{de}
MNF-MAS-Tricho24	65.41 ^{bcdef}	74.06 ^{bcdefg}	58.22 ^{bcde}	69.27 ^{bcde}	57.87 ^{bc}	70.08 ^{bc}	47.84^{bc}	54.63^{bcd}
Tricho25	65.57 ^{bcdef}	74.18 ^{bcdefg}	56.84 ^{bcde}	68.25 ^{bcde}	58.48 ^{bc}	70.51 ^{bcd}	82.08^{ij}	84.41 ^{ij}

* Values within a column followed by the same letter(s) are not significantly different at the P=0.05 level according to Duncan's multiple range test.

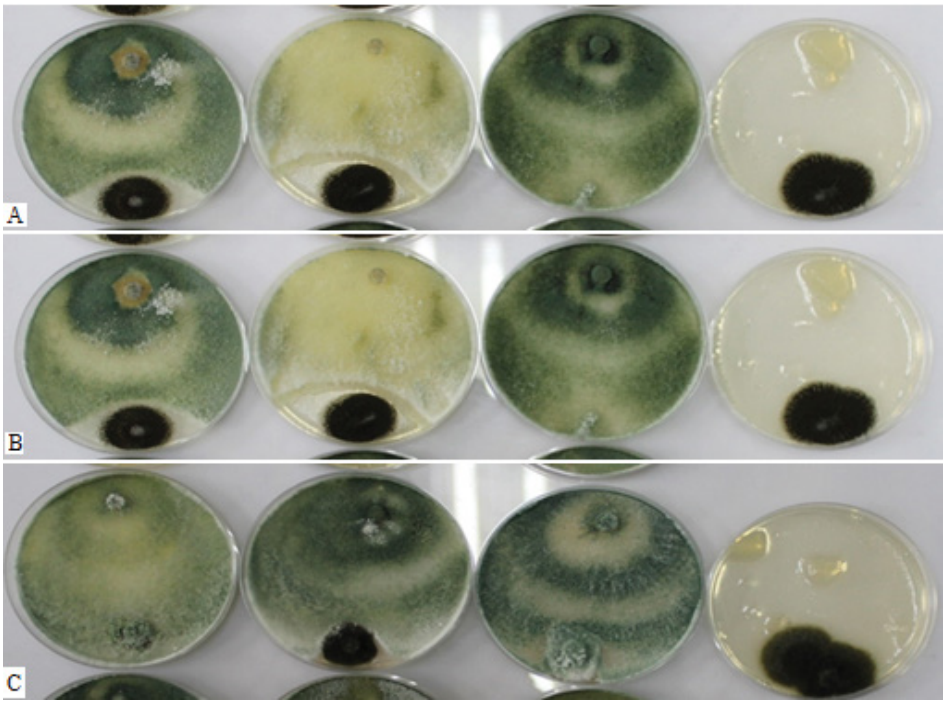

Figure 5: Dual-culture assay for in vitro inhibition of mycelial growth of various pathogenic fungi by *Trichoderma* strains on potato dextrose agar medium. (A) *Fusarium oxysporum* (B) *Sclerotium spp* and (C) *Alternaria alternate*.

Table 7: Classification of *Trichoderma* strains and isolates based on antagonism against different pathogen fungi after five and seven days of incubation.

Strain/isolate	*Alternaria alternata*		*Fusarium oxysporum*		*Rhizoctonia solani*		*Sclerotium spp.*	
	After 5 days	After 7 days	After 5 days	After 7 days	After 5 days	After 7 days	After 5 days	After 7 days
MNF-MAS-Tricho1	M	M	A	A	M	M	A	A
MNF-MAS-Tricho2	M	M	A	M	M	M	A	A
MNF-MAS-Tricho3	M	M	A	M	M	M	A	A
MNF-MAS-Tricho4	A	M	M	M	M	M	C	C
MNF-MAS-Tricho5	A	M	A	M	M	M	C	C
MNF-MAS-Tricho6	M	M	A	M	M	M	A	A
MNF-MAS-Tricho7	M	M	M	M	M	M	M	M
MNF-MAS-Tricho8	M	M	A	M	M	M	A	M
MNF-MAS-Tricho9	M	M	A	A	M	M	A	A
MNF-MAS-Tricho10	M	M	A	M	M	M	A	A
MNF-MAS-Tricho11	M	M	A	M	M	M	M	M
MNF-MAS-Tricho12	M	M	A	A	M	M	M	M
MNF-MAS-Tricho13	M	M	A	M	M	M	A	M
MNF-MAS-Tricho14	M	M	A	M	M	M	A	A
Tricho15	C	M	M	M	M	M	M	M
MNF-MAS-Tricho16	M	M	C	C	M	M	C	C
MNF-MAS-Tricho17	M	M	M	M	M	M	C	C
MNF-MAS-Tricho18	M	M	A	A	M	M	C	C
MNF-MAS-Tricho19	M	M	A	M	M	M	M	M
MNF-MAS-Tricho20	M	M	A	A	M	M	M	M
Tricho21	M	M	A	A	M	M	C	C
MNF-MAS-Tricho22	M	M	A	A	M	M	C	M
MNF-MAS-Tricho23	M	M	A	A	M	M	A	A
MNF-MAS-Tricho24	M	M	A	M	M	M	A	M
Tricho25	M	M	A	M	M	M	A	A

A, Class 1 (Antibiosis): Biocontrol agent grew and stopped without contact with the colony of pathogen and a zone of growth inhibition exists between the fungi.
C, Class 2 (Competition) Biocontrol agent grew and stopped without contact with the colony of pathogen.
M, Class 3 (Mycoparasitism) Biocontrol agent grew and overlapped the colony of pathogen.

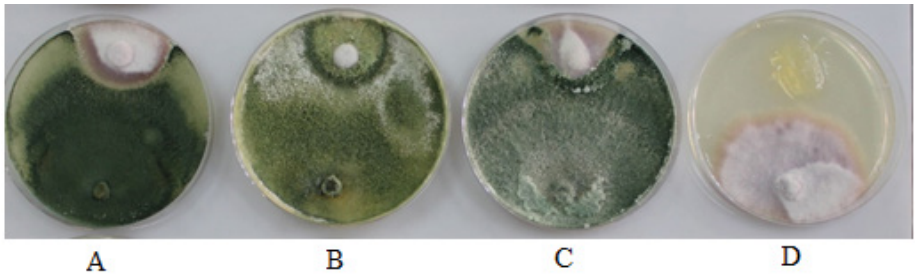

Figure 6: Different mechanisms of *Trichoderma* strains antagonisms. (A) Antibiosis (B) Mycoparasitism (C) Competition (D) Control pathogen only.

morphology serves to identify fungi of this genus, it is insufficient to distinguish the species, which makes it necessary to confirm the species through molecular methods [22,5]. Molecular methods are among the most precise tools used for differentiating species and identification of new strains. Specifically, comparison at the DNA sequence level provides accurate classification of fungal species and is beginning to elucidate the evolutionary and ecological relationships among diverse species [37]. Therefore 22 isolates were identified at the species level by using ITS sequences. Also, the analysis using TEF1-α sequences was able to confirm species identification of ten isolates that were differentiated using the ITS. Consequently, methods of identification of the isolates in this study yielded three species *T. harzianum*, *T. longibrachiatum* and *T. asperellum*, of which *T. harzianum* was the most frequently sampled. The presence of *T. harzianum* had already been reported in this region of the country [16] and it is the species with the widest distribution [8]. Also, these results agreed with [38] results who found only one species *T. harzianum* and [16] who found that the two species *T. harzianum*, *T. longibrachiatum* were predominant in Delta area of Egypt which includes Menoufia. Also, [39] who demonstrated that *T. harzianum* is predominant of samples obtained from three governorates including Menoufia and [40] found same results. In addition, [41] found only two species *T. harzianum* and *T. longibrachiatum* from samples obtained from

Table 8: Diameter of the colored zone on solid medium, and total chitinolytic activity and exochitinase activity in culture filtrate of *Trichoderma* strains and isolates.

Strain/isolate	Zone diameter* (mm)	Total chitinolytc activity (units/ml)	Exochitinae activity (u/ml×10 $^{-3}$)	Protein content (µg/ml)
MNF-MAS-Tricho1	65.00^{bc}	6.15	0.0137	240
MNF-MAS-Tricho2	68.20^{cd}	5.92	0.0124	40
MNF-MAS-Tricho3	78.13^{fghi}	4.21	0.0078	170
MNF-MAS-Tricho4	69.49^{cde}	2.06	0.0057	260
MNF-MAS-Tricho5	83.63^{j}	4.88	0.0102	70
MNF-MAS-Tricho6	76.27^{fgh}	5.25	0.0123	110
MNF-MAS-Tricho7	83.13^{ij}	4.58	0.0114	140
MNF-MAS-Tricho8	74.00^{ef}	7.10	0.0215	110
MNF-MAS-Tricho9	79.73^{ghij}	4.11	0.0.95	180
MNF-MAS-Tricho10	0.00^{a}	3.93	0.0117	250
MNF-MAS-Tricho11	61.43^{b}	6.18	0.0138	295
MNF-MAS-Tricho12	77.26^{fgh}	7.72	0.0256	95
MNF-MAS-Tricho13	73.17^{def}	7.00	0.0196	225
MNF-MAS-Tricho14	0.00^{a}	7.76	0.0245	315
Tricho15	73.07^{def}	7.78	0.0234	125
MNF-MAS-Tricho16	67.13^{c}	3.82	0.0119	165
MNF-MAS-Tricho17	75.10^{fg}	6.03	0.0148	195
MNF-MAS-Tricho18	75.27^{fg}	5.08	0.0127	165
MNF-MAS-Tricho19	81.13^{hij}	1.65	0.0068	235
MNF-MAS-Tricho20	83.70^{j}	7.13	0.0198	305
Tricho21	60.03^{b}	6.34	0.0145	125
MNF-MAS-Tricho22	0.00^{a}	2.33	0.0078	215
MNF-MAS-Tricho23	76.33^{fgh}	4.70	0.0115	25
MNF-MAS-Tricho24	0.00^{a}	3.48	0.0098	65
Tricho25	0.00^{a}	1.04	0.0005	95

* Values within a column followed by the same letter(s) are not significantly different at the P=0.05 level according to Duncan's multiple range test.

south Egypt. Finally, laboratory nomenclature strains were given to the 22 identified isolates. Phylogenetic tree of the 22 strains confirmed that the two strains *T. longibrachiatum* and *T. asperellum* came together in the same branch while the rest of the strains which were *T. harzianum* were on the other side of the tree.

The antagonistic effects of all *Trichoderma* strains and isolates were determined through the average inhibition percentage of mycelial growth of pathogenic fungi *Alternaria alternate*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Sclerotium spp.* This approach was frequently used and shown to be a useful way in assessing the antagonistic potential of the antagonistic fungi [39,41-43]. The results indicated that all *Trichoderma* strains and isolates exhibited inhibition to the mycelial growth against the four pathogens prior to mycelial contact. This could be due to their faster growing than the pathogenic fungi and competing efficiently for space and nutrients. In other

words, the competition for nutrients resulted in growth inhibition [44]. Another explanation is could be due to their production of diffusible components, such as lytic enzymes or water-soluble metabolites [45]. These diffusible components such as chitinases and glucanases were always secreted by *Trichoderma* in low level, so that they can act against the pathogenic fungi before mycelial contact [16]. It was found that the hyphae of pathogenic fungi were degraded by lytic enzymes produced by biocontrol strains, resulting in retardation of growth of pathogenic fungi [46]. Also, it was documented that the degraded cell wall components from pathogens induced the expression of genes allowing *Trichoderma* to be more antagonistic [10]. The mechanisms of antagonistic activity of *Trichoderma* against pathogen fungi; competition, antibiosis and mycoparasitism were well described [47]. The results indicated that competition mechanism was used mainly by *Trichoderma* strains and isolates against *Sclerotium spp.* fungi which may be due to that *Trichoderma* is fast growing than *Sclerotium spp.* Also, results indicated that *Trichoderma* strains and isolates antagonize by antibiosis mainly against *Fusarium oxysporum* and partially against *Sclerotium spp. Trichoderma* stop pathogen growth without physical contact and form a zone of inhibition without growth between pathogen and *Trichoderma* which could be due to secondary metabolite excretion such as viridin and its derivatives, which function as antimicrobials [48-50,10]. In addition, results indicated clearly that *Trichoderma* strains and isolates used mycoparasitism mechanism against *Rhizoctonia solani* where they overlapped pathogen and formed Hook-like and appressorium-like structures to penetrate the host. This may be due to mainly that *Rhizoctonia solani* cell wall forming mainly of chitin [47].

Many cell wall degrading enzymes are involved in antagonistic activity in *Trichoderma spp.* such as chitinase, gluconase, xylanase etc. However, chitinase enzymes are of great importance as fungal cell wall made up of chitin that why chitinolytic enzymes degrade phytopathogenic easily [51]. Studies indicated that chitinases are the key enzymes in antagonistic process [52]. Chitinolytic system of *Trichoderma* comprises of many enzymes and they are divided into 1,4 β- acetylglucosaminidases, endochitinases and exochitinases. These enzymes secreted by *Trichoderma spp.* when grew with colloidal chitin as sole carbon source, indicating the role of mycelia of pathogen as a stimulator of these enzymes' syntheses. Also, [53] observations showed that *Trichoderma* secreted elevated levels of chitinases when grown in medium having colloidal chitin and it was the best inducer of chitinase enzymes in comparison to other sources of chitin. Results of this research showed that strains and isolates formed purple colored zones after incubation with colloidal chitin in agar medium, indicating that they exhibited chitinase activities. These results clearly demonstrated that this method of selection the high chitinolytic strains of *Trichoderma spp.* was easy and fast [54]. Agrawal [14] stated that the soluble substrate with pH indicator dye bromocresol purple for the assay of chitinase activity on solid media is sensitive, easy, reproducible and economic option to determine chitinases. Therefore, it considered a fast and accurate one-step process for the selection of chitinolytic *Trichoderma spp.* [55-56]. Therefore, to confirm results, spectrophotometric determination of total chitinolytic activity of *Trichoderma* strains and isolates was conducted. Results clearly elucidated differences among strains and isolates in chitinases activities. This differential ability in producing of chitinases enzymes could be possibly because of the differential expression of certain genes in different *Trichoderma* strains [57]. In addition, these results were similar with those of [54] and [14]. However, it was found by our results that some of the strains did not show any response in agar medium test and they showed some values in spectrophotometric method and also there was that no correlation between diameter measurements and chitinolytic activity quantified spectophotmerially. This phenomenon of disagreement may be due to the differences in sensitivity of the two assays and also, it might be due to the differences of the quantifying the enzyme secreted in liquid medium and in agar plates. Also, results of this study showed no correlation between chitinolytic activity and percentage growth inhibition after five and seven days for all isolates and strains against the four pathogens understudy. These latest results were also reported by [58,59,14] and the possible explanation was that that the production of the hydrolytic enzymes has been affected by culture conditions and by the host.

Finally, exochitinase activity (N-acetyl-β-D-glucosaminidase) was measured as release of p-nitrophenol (pNP) from p-nitrophenyl-N-acetyl-β-D-glucosaminide (pNPg). Different values were obtained from different *Trichoderma* strains and isolates and highly positive correlation (0.93) was found between the values of total chitinolytic activity and exochitonolytic activity of the 25 strains and isolates. It was obvious to find this direct relationship because exochitonolytic activity is part of the total chitinolytic activity. Finally, total protein contents of strains and isolates were measured, and no correlation with enzyme activity was found. Possible explanation that *Trichoderma* may produce large amount of proteins and not all of them are enzymatic proteins. Ahmed [60] mentioned that *Trichoderma* biomass protein produced by this fungus can be used as rich source of protein. Rey [61] explained that the increase amount in secreted proteins by the *T. harzianum* could be related to post-translational events such as those related to secretory pathways and/or membrane permeation. Also, [62] showed that the presence of *Rhizoctonia solani* cell walls enhanced secretion of biocontrol enzymes by *Trichoderma harzianum*.

CONCLUSION

T. harzianum species is the predominant species in Menoufia Governate of Egypt. Some of the isolated strains are potential to be used as biocontrol agents.

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