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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2017 14(4): 1156-1166. **OPEN ACCESS**

Identification and molecular characterization of egyptian *trichoderma* isolates

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Phytopathogen fungi are posing serious problems to the worldwide cultivation systems of economically important plants. Eco-friendly bio-control agents become necessary to help in resolving this problem. In this study, soil and rhizosphere samples were collected from five locations in Egypt. While forty three *Trichoderma* isolates were successfully isolated*.* Of these forty three fungi, fourteen isolates showed antifungal activity, six of them were belonged to *Trichoderma harzianum*. The other eight isolates included *T. asperellum*, *T. viride*, *T. hamatum* and *T. lixii*. The effect of identified isolates of *Trichoderma spp.* on radial growth and the percentage of inhibition toward the different fungal pathogens were studied in dual culture on Petri dishes. The molecular identification of the isolated *Trichoderma* spp*.* was done using two taxon-selective primers (ITS1 & ITS4) for the internal transcribed spacer (5.8S-ITS) region in the nuclear ribosomal repeat unit. The sequence BLAST analysis of the ITS sequences showed that the isolates were belonging to *Trichoderma* spp*.* The phylogenetic relationship was conducted between isolated sequences. Full length of chitinase gene (1.2 Kb) was isolated from two different species and comparatively analyzed between other isolates of high antifungal activity. Select the best medium in terms of growth spore production and biomass yield for the production of *Trichoderma* spp.

Keywords: ***Trichoderma*, biocontrol agents, internal transcribed spacer.**

INTRODUCTION

One of the most important aims of the green revolution in agriculture biotechnology development is providing enough food to feed the world`s rising population. The major serious problems are the increasing human population, the erosion of agricultural land and the losing of valuable percentage of crops through the plant diseases and pathogens (Kumar and Gupta, 2012).The term biological control means the utilization of some biological material to control the biological causatives plant diseases and to increase crop productivity (Chet, 1990). Biocontrol agent is considered economically suitable and safe (Jones and Stewart, 2000 and Chernin and Chet, 2002). Currently, some biocontrol agents are prepared and marketed from *Trichoderma* spp. (Fokkema, 1996). *Trichoderma* spp. acts against fungi plants (Hokkanen, 1994). Some of the ways *Trichoderma* uses to control fungi are antagonism, parasitism, producing antibiotic and lysis enzymes, competition for nutrient and space, and induction of host resistance (Pal and Gardener, 2006). *Trichoderma* is the most famous fungi in the world due to their prevalence in all latitudes worldwide (Smith and Read, 2008). Their genome size ranged from 36.1 Mb to 40.98 Mb (Druzhinina et al, 2011). It has fast growth rate, production of large amount of spores, beneficial products and associate with plant roots (Howell, 2003). Therefore, *Trichoderma spp.* can be the most common biofungicides to control plant

diseases and also in symbiosis with plants (Mohamed et al, 2010 and Lorito et al, 2006). The presence of chitin in nature is very high rating (Tokimoto, 1982). Chitinase produced by *Trichoderma spp.* can be used as a bio control agent against Pathogenic plant fungi (Kubicek et al, 2001). Several chitinolytic enzymes were found in *T. harzianum* (De la Cruz, 1992).

The purpose of this study is to investigate, identify and characterize variation in some egyptian *Trichoderma* isolates with chitinase activity uses as environmentally safe alternatives to protect local crops from fungal diseases.

MATERIALS AND METHODS

Isolation of *Trichoderma* spp. from soil

Forty three soil samples from different cultivated fields of five different localities in Egypt were collected. The dilution plate method was used for the isolation of *Trichoderma* spp according to (Gams *et al.,* 1987). Plate was then incubated at 28°C and daily checked for the developing colonies (7- 10 days). Based on the appearance, *Trichoderma* colonies were transferred to Potato Dextrose Agar (PDA) plates till pure cultures were obtained. All the confirmed *Trichoderma* cultures were maintained on PDA at 4°C in a refrigerator and revived monthly.

Morphological and molecular characterization of the isolated fungi

After seven days of pure culturing on PDA, the developed fungal colonies were microscopically examined using the morphological characteristics like growth rate, color, texture, rate of sporulation (Barnett, 1960 and Domsch *et al*., 1980). Cultures were multiplied and maintained on PDA slants and stored at 4ºC. The isolation frequency of each isolate was recorded based on soil sample location. The molecular identification of isolated *Trichoderma spp*. was done by using two taxon-selective primers for the internal transcribed spacer region in the nuclear ribosomal repeat unit. PCR reaction conditions were: 94º C for 4 min; 35 cycles of denaturation at 94º C; annealing at 56º C; and extension at 72º C for 1 min each, followed by 7 min final extension at 72º C.

Screening of Trichoderma spp.

Based on antagonism against fungal plant pathogens

The antagonism experiments were done as described by (Goes, et al. 2002). The fungal isolates were cultivated using paired growth of colonies on PDA media in Petri dishes (9-cm-diameter) with PDA for seven days. In each plate, two fungi, one of *Trichoderma spp*. and one of plant pathogen (*Fusarium oxysporum* or *Fusarium solani*) were placed opposite to each other, in regard to the growth speed of the two fungi. The plates were incubated at 28°C. The experiment was repeated with three replications and observed at 12 h interval times for 7 days.The percentage of inhibition (I %) on the mycelial growth of *F. oxysporum* and *F. solani* were calculated using this formula:

$I \% = {}^{(r1 - r2)} / {}_{r1}$

Where r1 is the radius of Fusarium away from Trichoderma isolate, while r2 is the radius of Fusarium towards Trichoderma isolate (Abadi, 1990).

Bacterial strains and plasmids

Escherichia coli of strain JM109 was used as host cells for all transformation experiment. The pGEM-T easy (Promega) system was used to clone the *Trichoderma* chitinase gene. PCR reagents, T4 DNA ligase and restriction endonuclease were purchased from New England Biolab Inc., USA. *E. coli* cells with plasmids were grown aerobically in LB (Luria Bertani) liquid medium or solid medium at 37 °C, supplemented with Ampicillin (100ug/ ml) for the selection of transformants.

Isolation of chitinase gene from *Trichoderma*

Chitinase gene corresponding to 1.2 Kb fragment size was amplified using forward primer (Tri- F) 5-GCTCTAGA ATG CCTTCATTGACTGCTCTTGCG -3 and reverse primer (Tri- R) 5- CGTCTAGA TTA CCTCAAAGCATTGACAACC -3. The conditions for PCR were 94 °C, 1min; annealing at 56 °C, 1.5min; and extension at 72 °C, 1min for 30 cycles, followed by 7 min final extension at 72º C.

Cloning of chitinase PCR fragments into pGEM®-T Easy vector

PCR products of chitinase gene amplification were purified using QIAquik®PCR Purification system, then used for cloning into pGEM®-T Easy vector. To prepare ligation mixture the following components were added, 1µl 10x of ligation buffer, 2µl of cloning pGEM®-T Easy vector (50 ng), 1µl of T4 DNA ligase, (300-500 ng) of purified PCR product (3-5 molar excess), and ddH$_2$o up to 10µl. Components were mixed briefly and shortly spined down to collect the contents in the tube.

Ligation mixture was then incubated at 4° C for ~16 h.

Transformation into *E. coli*

Ligation mix (5µl) was added to 100µl of competent cells mixed gently by tapping and incubated on ice for 30 min. Transformation mixture was transferred to a 42°C water bath for exactly 40 sec then moved directly to ice and incubation continued for additional 2 minutes. An aliquot of 500 µl of L.B broth medium was added to each tube and mixed gently. Incubation was performed at 37° C with moderate shaking for one hour. The LB agar plates supplemented with ampicillin were prepared. For blue/white transformants screening, 40 µl of ×-gal (50 mg/ml) and 4µl of IPTG (1 M) were added to each agar plate. About 50 µl of transformed cells were added to each plate, uniformly spread, and incubated at 37° C for 18-24 h.

Plasmid DNA preparation and digestion

Plasmid DNA was prepared from *E. coil* host cells, using the Wizard plus DNA purification system (Promega). About one microgram of plasmid DNA was digested using *EcoRI* restriction enzyme in 20 µl total volume of 1× *EcoRI* buffer. Digestion reaction was incubated at 37˚C for 2 hours. Size confirmation of digestion was visualized using agarose gel electrophoresis system (BioRad).

DNA Sequencing

DNA sequencing was carried out using the automated DNA sequencing method. Briefly, the automated DNA sequencing reactions was performed with ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, USA) using the ABI PRISM (310 Genetic Analyzer).

RESULTS

Isolation of *Trichoderma* spp.

Trichoderma isolates were identified as *T. harzianum*, *T. hamatum*, *T. viride*, *T. asperellum*, and *T. lixii*. These five species represent and comprise fourteen different isolates were originated from different localities as follows:

T. harzianum was the most frequent specie represented by six isolates obtained from localities (Gamasa (Dumyat); Nubariya (Beheira); El-Kasassin (Ismailia); and El-Kanater (Qalubiya). *T. asperellum* was represented by four isolates from (Baltim (Kafr El-Sheikh); El-Kanater (Qalubiya). *T. viride* was represented by two isolates [Baltim (Kafr El-Sheikh)]. *T. lixii* was represented by one isolate El-Kasassin (Ismailiya). *T. hamatum* was represented by one isolate Nubariya, (Beheira).

Morphological Characterization

Colony characteristics

Colony characteristics of *Trichoderma* isolates were studied using seven days old PDA cultures. All *Trichoderma* isolates were grown well and formed conidia within 4 days. The conidial production in *T. viride* was diffused, dispersed and formed concentric rings. The color of mature conidia in *T. viride* was light green. For *T. lixii*, the mature conidia appeared to have grayish green color, and no concentric rings were observed. The conidia of *T. lixii* were tending to concentrate at the center of the colony. For isolates *T. asperellum* and *T. hamatum*, dark green color of conidia were tend to form in pustules, and arranged in concentric rings. More concentric rings were observed in these colonies compared to that in colonies of *T. viride*. In the colonies of T. *harzianum*, however, no concentric rings were observed. Their conidial productions were restricted to the center of the colonies, diffused, and appeared to be yellowish green color.

Growth rate

Nearly all isolates of *Trichoderma* on PDA at 28°C, had the same growth rate except the isolates of *T. harzianum* and *T. hamatum*. *T. harzianum* grew faster than any other isolates at 28°C on PDA. The fast growth rate of *T. harzianum* was more obvious after 2 days. However, *T. hamatum* grew slightly slower than any other isolates at 28°C on PDA and fully colonized as nine cm as vented PDA plate at day four. All isolates except *T. hamatum* were fully colonized on PDA plates at day three.

Microscopic features

The microscopic features of *Trichoderma* isolates were observed under light microscope with 400X magnification power. The color of conidia of most *Trichoderma* was green. The different intensities of green color (light, yellowish, dark and grayish) of mature conidia observed on PDA plate were hardly observed under light microscope. Both *T. viride* and *T. hamatum* had ellipsoidal conidia. However, the conidia of *T. viride* were wider than those of *T. hamatum*. On the other hand, the isolates *T. asperellum* had more subglobose than globose conidia. The

conidial sizes of these isolates were almost similar, except the conidia size of *T. asperellum*, which tends to be slightly smaller than conidia of other isolates. Different from other isolates, only globose conidia were observed in *T. harzianum* and *T. lixii*.

Antagonistic activity of *Trichoderma* in dual culture

The effect of different isolates of five *Trichoderma spp*. i.e. *T. harzianum*, *T. hamatum*, *T. asperellum*, *T. lixii* and *T. viride* on radial growth and the percentage of inhibition toward the different pathogenic fungi was studied in dual culture in Petri dishes on PDA at 28°C for 5 days . Different isolates of each *Trichoderma spp.* were tested individually against plants pathogens diseases i. e. *F. solani* and *F. oxysporum*. In all the dual culture plates tested, the contact zone was a curve, with concavity oriented towards the pathogenic fungi. In the negative control plates, only *Fusarium* species were inoculated. The averaged inhibition percentage (I %) of mycelial growth for *F. oxysporum* and *F. solani* were presented in Table (1). Results given in Table (1) showed significant differences in radial growth of the different pathogens tested. Also, there was a significant difference between the pathogens in dual culture and the control treatment which include the pathogen without biocontrol agent. All *T. harzianum* isolates showed percentage of radial growth inhibition reached less than 70% with different pathogens. *T. harzianum* isolate (TZ4) showed the lowest percentage of radial growth inhibition with *F. oxysporum* (27.76%). On the contrary TZ4 and TZ5 showed the highest percentage of inhibition (57.64 and 52.22%, respectively) with the pathogen *F. solani*. All isolates of *T. harzianum* showed moderate effect on the percentage of radial growth inhibition with other pathogens. On the other hand, *T. lixii* and *T. viride* isolates showed the highest percentage of inhibition reached more than 50% in many cases, and TL1 showed 61.14% and TV1 showed 64.63 respectively with *F. oxysporum* as well as they showed 60.27% and 56.06 with *F. solani*. Two isolates of *T. asperellum* showed moderate effect on the inhibition of the pathogens growth which showed the high effect on *F. oxysporum*, where TA1 and TA2 showed 54.30 and 56.05%, respectively. But the percentage of inhibition was lower with the other pathogens; it reached 35.40 and 40.89% on *F. solani*. On isolate of *T. hamatum* (Th1) showed moderate effect on the inhibition of the pathogens growth. It showed inhibition percent started from 28.64% on *F. oxysporum* and 35.26% on *F. solani*.

PCR amplification of 5.8S-ITS region

The morphological identification of *Trichoderma* isolates from the Egyptian rhizosphere was complemented with a molecular methods based on internal transcript spacers (ITS region) of 18S rRNA sequences. The genes encoding ribosomal RNA are organized in arrays, which contain two repetitive intergenic transcriptional spacers ITS1 and ITS4, involving 16-18S, 5.8S and 23-18S units. These units are transcribed by RNA polymerase I and separated by non-transcribed intergenic spacer (IGS). The product of RNA polymerase I is processed in the nucleolus, where the ITS1 and ITS4 are excised and three types of rRNA are produced. Six *Trichoderma* isolates out of total 43 fungal isolates were selected for PCR analysis of rDNA genes including the 5.8S and the flanking ITS regions.

Table 1. Average inhibition percentage of mycelial growth by different *Trichoderma* isolates.

Trichoderma isolate	Average percentage of inhibition against F. oxysporum (%)	Average percentage of inhibition against F. solani (%)
TZ1	66.67	65.52
TZ2	40.00	25.00
TZ3	52.63	38.46
TZ4	27.76	57.64
TZ5	35.71	52.22
TZ6	39.29	58.62
TL1	61.14	60.27
TV1	64.63	56.06
TV2	54.29	51.85
TA1	54.30	35.40
TA2	56.05	40.89
TA3	43.33	52.94
TA4	60.00	48.00
Th1	28.64	35.26

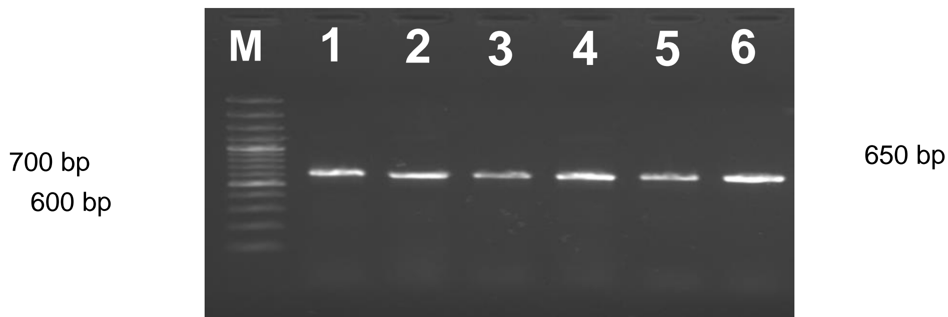

Figure.1. 650 bp 5.8S-ITS region amplified for all *Trichoderma* isolates using primer pair ITS1 and ITS4. Lane (M) 100 bp DNA ladder, lane (1) TZ1 isolate, lane (2) TA1 isolate, lane (3) TA2 isolate, lane (4) TA3 isolate, lane (5) TA4 isolate and lane (6) TL1 isolate.

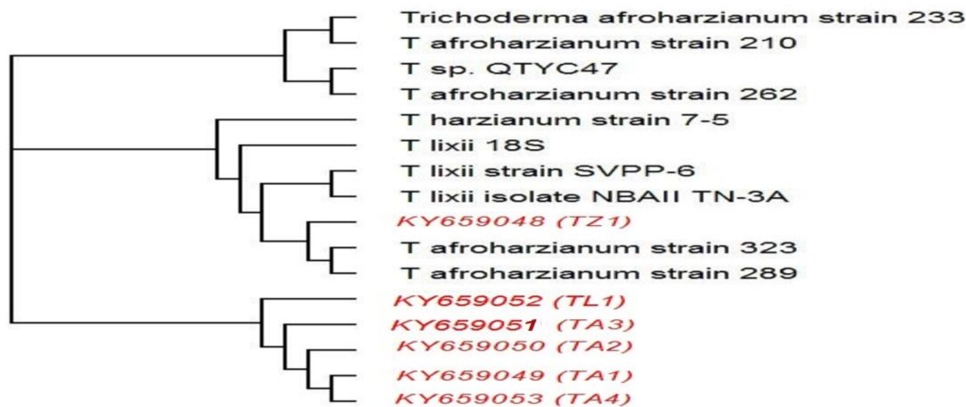

Figure. 2. Phylogenetic tree based on the sequences of *Trichoderma* isolates using primer ITS1.

Amplification and cloning of chitinase genes

These isolates were selected based on their antagonistic activities, modes of action and their determined of chitinase activities.

Genomic DNA was prepared from the six isolates of *Trichoderma* and used to screen primers for reliability and reproducibility of PCR amplification.

The employed PCR conditions allowed amplification for single fragment appearance on the agarose gel. Amplification of the ITS using primers ITS1 and ITS4 yielded a single fragment at size of approximately 650 bp (Figure 1).

Sequencing and phylogenetic analysis of 5.8S-ITS region

PCR fragment that were amplified from the different *Trichoderma* isolates were sequenced. Fragments were aligned and a consensus sequence was generated from each alignment, BLAST program was used to determine the species identity of *Trichoderma* isolates. The resulted sequences were submitted to GenBank database and accession numbers were obtained in Table (2). The BLAST results showed one isolate (TZ1) as *T. harzianum*, four isolates (TA1, TA2, TA3 and TA4) as *T. asperellum*, while another isolate (TL1) as *T. lixii*. The Phylogenetic tree obtained for sequence analysis for our *Trichoderma* isolates and others *Trichoderma* obtained from NCBI, GenBank was represented in (Figure 2).

Table 2.BLAST search results for *Trichoderma* isolates.

Isolate code	Accession no.	Species identified
TZ1	KY659048	*T. harzianum* strain 7-5
TL1	KY659052	*T. Lixii* isolate MR42-1
TA1	KY659049	*T. asperellum* isolate SMZC
TA2	KY659050	*T. asperellum* isolate CRT2
TA3	KY659051	*T. asperellum* strain SVPP-3
TA4	KY659053	*T. asperellum* isolateTB3

Amplification and cloning of chitinase genes

The chitinase gene (1.2 Kb) of *Trichoderma* isolates was amplified from genomic DNA using the specific primers Tri-Chi-F and Tri-Chi-R based

on sequences of existing chitinase present in the database (Figure 3). The recombinant plasmids were transformed into *E. coli* strain (*JM109*) competent cells. Two clones harboring the recombinant plasmid pCHI were selected for further analysis. The recombinant plasmid pCHI were extracted, purified and subjected to nucleotide sequences.

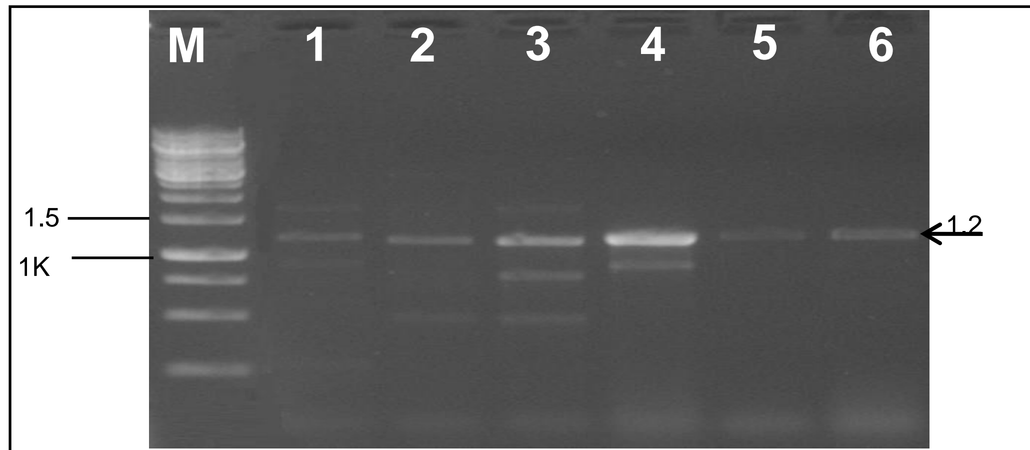

Fig. 3. PCR amplification of chitinase gene with specific primers from *Trichoderma* isolates. Lane (M) 1Kb plus DNA ladder, lane (1) TZ1 isolate, lane (2) TA1 isolate, lane (3) TA2 isolate, lane (4) TA3 isolate, lane (5) TA4 isolate and lane (6) TL1 isolate.

Confirmation of clones

Two clones were selected; one clone from (TZ1) and the other from (TA1). The two selected clones were subjected to restriction digestion using EcoR1 to test for the presence of chitinase coding sequence. The cloning vector pGEM®-T Easy has a two EcoR1 sites (nucleotide 319) and the chitinase gene has not EcoR1 sites. Accordingly, two clones were shown to harbor the presence of chitinase gene in the cloning vector pGEM®-T Easy (Figure 4). The PCR and restriction analysis of selected clones proved the chitinase gene size, then subjected to final analysis confirmation sequencing. Sequence analysis indicated that the chtinase gene consists of an open reading frame of 1200 nucleotides, which encodes a protein of 400 amino acid residues. Amino acid sequence analysis revealed that chitinase from TZ1 and TA1 has, respectively, 94% and 96% similarity with chitinase sequences from *Trichoderma harzianum* strain 7-5 and *Trichoderma asperellum* isolate SZMC 20787, as well. The chitinase was analyzed by SignalP program (http://www.cbs.dtu.dk/services/SignalP) for the prediction of a possible signal sequence.

Amino acids analysis of chitinase

Sequence analysis revealed that the N-terminal sequences of chitinase gene contained numerous hydrophobic residues, characteristic of a leader peptide or a signal sequence. In addition, a putative signal peptidase cleavage site between Gly_{23} and Ser_{24} was predicted.

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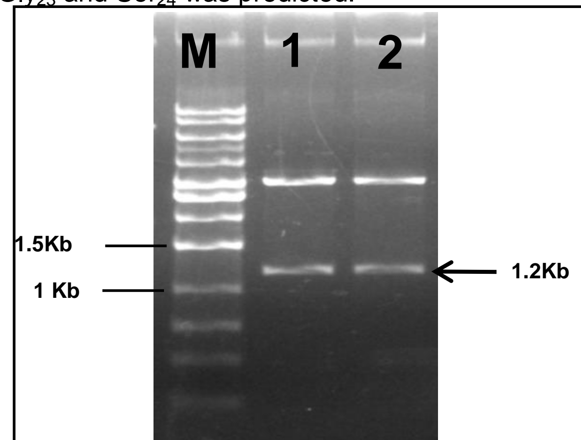

Fig. 4. Restriction digestion of recombinant clone using restriction enzyme with EcoR1 lane (1) chitinase derived from TZ1 isolate , lane (2) chitinase derived from TA1 isolate and lane (M) 1Kb plus DNA ladder.

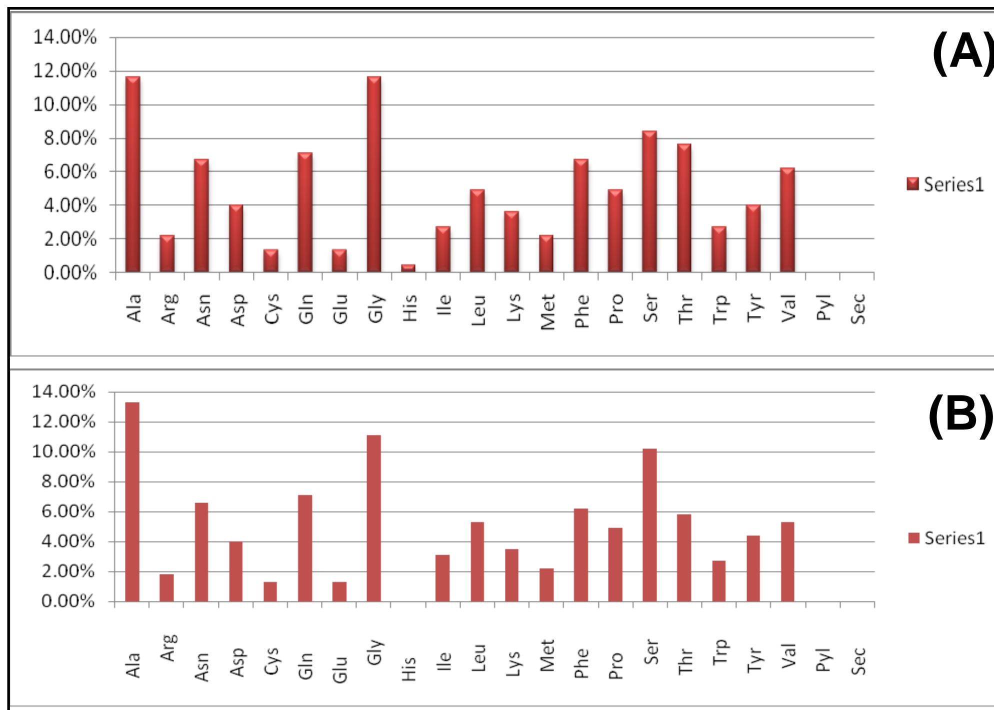

Figure. 5. Comparison of percentages of deduced amino acids between chitinase genes of (A) TZ1 isolate and (B) TA1 isolate.

Together, these suggested that chitinase gene is a secretory protein. The chitinase ORF consists of 1200 bp and code for 400 amino acids. Secondary structure prediction using the Jpred 3 server (http://www.compbio.dundee.ac.uk/www-jpred) revealed that chitinase consists of a α-helix-1 (Ser_5-Asn_{32}), a α-helix-2 (Thr_{47}-Ala55) and a β-strand (Phe_{64}-Cys_{68}). The chitinase gene has a signal peptide (amino acid 1- 45), most of α-helix-1, and another signal peptidase recognizing site between Phe_{45} and Gly_{46}. The chitinase gene has nine cysteine residues (Cys_{50}, Cys_{72}, Cys_{123}, Cys_{135}, Cys_{202}, Cys_{275}, Cys_{355}, Cys_{375} and Cys_{377}), represent 1.3% of amino acid content. The presence of cysteine residues is very crucial for disulfide bond formation and protein stabilization. The three dimensional structure of chitinase gene was constructed using the SWISS MODEL Workspace (http://swissmodel.expasy.org /workspace) and fungal chitinase 2 as a template. Both chitinase genes of *Trichoderma* and fungal chitinase 2 shared some amino acid similarities at the N-terminal region (Figure 5). Although the amino acid similarity was low between the two polypeptides, the secondary structure topology was very similar.

Study mycelia growth of *Trichoderma* on different culture media

Mycelia growth, conidial production and biomass yield of four different *Trichoderma* species (TZ1, TA1, TA2 and TL1) were examined in one liter flask on four different culture media including: Potato Dextrose agar, Waksman agar, Water agar medium, Corn steep liqur & soy flour corn steep (fermentation media), molasses-yeast extract medium (fermentation media), Coffee husk which is a waste from coffee curing industry, Peptone (5 gm), Mono potassium phosphate (1gm), Magnesium sulphate (0.5 gm) (media failed) and Banana waste urea (media failed). A 5 mm block of each isolate was placed at the centre of five replicated petri plates containing different culture media and incubated at 25°C with factorial arrangements. For biomass production Erlenmeyer flask (250 ml) containing 100 ml of each medium was inoculated with four mycelial plugs of the fungus taken from seven days old cultures on PDA. The flasks were plugged in aseptic conditions and placed in incubator at 30°C for 28 days. The culture was harvested finally from each replicate. The fungal biomass yield was assessed by collecting fungal biomass on

reweighed filter paper. The dry weight was determined after 24 hours of oven drying at 60°C. Number of conidia per mg of the biomass was determined by dilution method with the aid of haemocytometer.

Initial inoculum for inoculation of fermenter

The four *Trichoderma* spp. were grown on potato dextrose agar medium for seven days. Initial inoculum was prepared by scraping seven days old and fully sporulated cultures grown on PDA plate surface with sterile scalpel in sterile deionized water containing 0.1% Tween 20. The flasks were incubated at 28 ºC for 72h. The entire contents of the flasks were churned using a blender and one liter of initial inoculum was added to 10 liters of fermenter medium. The optimum fermentation conditions for obtaining maximum biomass of *Trichoderma* spp. in molasses-yeast broth were temperature 27ºC, pH 7.0, dissolved oxygen 40%, stirring rate 250 rpm for first 48h and 450 rpm for rest of the period. Silicon (0.1%) was used as an antifoam agent. Highest biomass of *Trichoderma* spp. was obtained in molasses yeast extract broth after 72 hours of fermentation.

DISCUSSION

Forty three *Trichoderma* isolates were identified from different samples collected from rhizosphere soil. Morphological characterization was conventionally used in the identification of *Trichoderma* species, and it remains as a potential method to identify *Trichoderma* species. Due to similar growth rate of *Trichoderma* isolates on PDA cultured at 28°C no much information to differentiate between isolates was observed. However, the ability of *Trichoderma* isolate to grow at 28°C is useful to identify *Trichoderma* species. In this study, *T. harzianum* isolate can be distinguished from other isolates due to its restricted growth at 28°C. Besides, *T. harzianum* showed the fastest growth rate compared to other isolates at 28°C. Thus, *T. harzianum* can be differentiated from other isolates based on its fast growth rate. In addition to macroscopic characteristics and growth rate, microscopic features of *Trichoderma* isolates were also important as morphological keys in the identification of *Trichoderma* species. The production of chlamydospores by all *Trichoderma* isolates also observed within 10 days. The appearance of chlamydospores was uniform and did not provide much information for the identification of *Trichoderma* isolates. The chlamydospores were unicellular, globose to subglobose, and usually formed on the hyphal tips. However, evaluation based on the presence or absence of chlamydospores within 10 days can be used as a morphological character for the identification of *Trichoderma* species. However, using the morphological evaluation alone is insufficient to precisely identify *Trichoderma* species, this due to the relatively few morphological characterstics and the limited variations that may cause overlapping and misidentification between the isolates. Besides, morphological characteristics are influenced by culture conditions. Therefore, there is a necessity to use other biological and molecular techniques to compensate for such limitations. The antagonistic capacities of all *Trichoderma* isolates against two major plant pathogenic fungi that reside in Egypt were investigated using dual culture method. The two fungal species tested in this study were *Fusarium solani* and *Fusarium oxysporum*. In all the dual culture plates, the contact zone appeared as a curve, with concavity oriented towards *Fusarium*. The curvature of the contact area between the colony of antagonistic fungi and the colony of pathogenic fungi in the same PDA plate depend on the growth rate of the colonies. If one colony has a faster growth rate than the other, a curve in the contact zone will most probably be observed. However, if the two colonies have the same growth rate, a straight line would be observed when mycelia from both fungi come into contact (Petrescu et al, 2012). Moreover, all *Trichoderma* isolates exhibited inhibition to the mycelial growth of both *F. oxysporum* and *F. solani* prior to mycelial contact. This could be due to the production of diffusible components, such as lytic enzymes or water-soluble metabolites, by Trichoderma isolates (Anees, 2010).

From the antagonistic test against *F. oxysporum*, all isolates were found to have inhibition on the mycelial growth of F. oxysporum, with the lowest inhibition percentage of 27.76% by TZ4 (*T. harzianum*), and highest inhibition percentage of 66.67% by TZ1 (*T. harzianum*). In the antagonistic test against *F. solani*, all isolates were also shown to inhibit the growth of *F. solani*. TZ2 (*T. harzianum*) established the lowest inhibition percentage, 25% whereas TZ1 (*T. harzianum*) showed the highest inhibition percentage, 65.52%. Overall, all *Trichoderma* isolates have the ability to inhibit the mycelial growth of *F. oxysporum* and *F. solani*. Nevertheless, TZ1 isolate was the most efficient isolate in inhibiting the mycelial growth of the

pathogenic fungi tested. It was also found that the antagonism of different isolates that belong to the same species varied. Isolates TZ1 and TZ4 which were both identified as *T. harzianum*, showed big difference in their inhibition percentage (26.67%). Similarly, this phenomenon was also observed among isolates TZ2, TZ3, TZ5 and TZ6. This phenomenon could be due to the different biological control mechanisms established by the isolates (Anees, 2010). Another possible explanation for this situation is that different isolates may have different efficiency in expressing the genes responsible for antagonistic activities. The isolates that can express these genes more rapidly and efficiently are usually better antagonists. DNA sequencing of the 5.8S-ITS region was carried out, by comparing the sequences of the 5.8S-ITS region to the sequences obtained from GenBank. All of the *Trichoderma* isolates were identified at the species level and homology percentage of 97%. Moreover , all six isolates were identified, and the results were in agreement with the BLAST results. Morphological and molecular approaches suggested important roles in the process of identification of *Trichoderma* isolates. Each approach has its own limitations and strengths. By combining morphological and molecular approaches, all *Trichoderma* isolates were successfully identified and confirmed. The results obtained from the morphological characteristics and BLAST approaches were in agreement. Among the six isolates, One isolate (TZ1) was identified as *T. harzianum*, four isolates (TA1, TA2, TA3 and TA4) were identified as *T. asperellum*, while another isolate (TL1) was identified as *T. lixii* were identified. The Phylogenetic tree obtained by sequence analysis of ITS1 and ITS4 of our *Trichoderma* isolates and the sequences of 10 *Trichoderma* spp obtained from NCBI, GenBank is represented in (Figure 2). The sequences of ITS region of the six *Trichoderma* isolates were submitted to GenBank under the following accession numbers (KY659048) for the *T. harzianum* isolate (TZ1), (KY659049, KY659050, KY659051 and KY659053) for the *T. asperellum* isolates (TA1, TA2, TA3 and TA4) and (KY659052) for the *T. lixii* isolate (TL1), respectively. The dendrogram showed two clusters; the first included the *T. harazianum* isolate. However, the second cluster included the *T. asperellum* and *T. lixii* isolates. This indicates the ability of the ITS similarity technique to differentiate between *Trichoderma* species. In conclusion, our results suggest that molecular identification is very important to identify the *Trichoderma* species and it must be used to confirm morphological approaches in the identification of *Trichoderma* isolates. Further, a full length chitinase gene from two isolates (TZ1) and (TA1), was amplified at fragment size of 1.2 kb that were separately cloned into pGEM®-T Easy vector. A variety of techniques were used to verify the cloning of the chitinase gene. These included Polymerase Chain Reaction (PCR) and restriction enzyme digestion, to confirm the identity of the recombinant cloned genes and the presence of the nucleotide sequences matching the chitinase gene. Two clones were obtained as one fom each isolate and sequenced at nucleotide level. The clones had 94% and 96% of homology as other reported chitinase from *Trichoderma harzianum* strain 7-5 and *Trichoderma asperellum* isolate SZMC 20787, while 100 % at amino acid level, respectively. Analysis of the sequence data revealed that the chitinase gene contained one large ORF of 1200 bp that extends from the start codon (ATG) through the stop codon (TAA), and encodes 400 amino acid residues (Figure 5). The molecular mass of the protein calculated from the deduced amino acid sequence was 44000 Daltons. The chitinase has a signal peptide (amino acid 1- 45), most of α-helix-1 and a signal peptidase recognizing site between the Phe_{45} and Gly_{46} residues. The chitinase gene had nine cysteine residues (Cys_{50}, Cys_{72}, Cys_{123}, Cys_{135}, Cys_{202}, Cys_{275}, Cys_{355}, Cys_{375} and Cys_{377}), almost 1.3% of amino acid content. The presence of cysteine residues is very crucial for disulfide bond formation and protein stabilization. The stability of chitinase gene is computed to be 81.85% and 82% for (TZ1) and (TA1), respectively. The three dimensional structure of chitinase was constructed using the SWISS-MODEL Workspace and Both chitinase gene of *Trichoderma*. Both chitinase and fungal chitinase 2 share some amino acid similarities at the N-terminal ends arginine. The study of mycelia growth on different culture media shows that, the medium had a significant effect on growth rate and population of the four Trichoderma species. The mycelial growth was measured after five days of inoculation. Average linear growth rate (ALGR) was calculated by the formula according to (Aneja, 1993). For biomass production, all the culture media used showed significant effect at (P=0.05) on mycelial growth, sporulation and biomass production of the *Trichoderma* species. The best medium was molasses-yeast agar supporting maximum growth rate (45.43 mm) and

minimum growth rate was observed on Corn liqur Agar medium (9 mm) for all the Biocntrol agents. TZ1 showed best growth rate utilizing the all culture medium followed by (TA1, TA2 and TL1). Significant interaction showed that maximum growth rate was observed in case of TL1 on molasses yeast medium and minimum growth was in case of (TZ1 and TA1) on Water agar medium. Similarly for spore production, maximum number of spores per 100 ml of culture media was produced by all the *Trichoderma* spp. on molasses yeast medium and minimum spore production was observed on Corn liqur agar medium. The spore counts was maximum on molasses yeast (16.05), Waksman agar medium (12.76), followed by corn liqr (10.23), water agar (10.73) and least number of spores obtained on Corn steep liqur Agar (5.64) for all the *Trichoderma* species. Maximum spore production capacity was in case of TZ1. Molasses yeast medium was the best medium in terms of growth spore production and biomass yield.

CONCLUSION

We investigate, identify and characterize variation in some Egyptian *Trichoderma* isolates with chitinase activity which uses as environmentally safe alternatives to protect local crops from fungal diseases.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

The author would thank all participants and their parents.

AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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