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Screening and identification of novel cellulolytic *Trichoderma* species from Egyptian habitats

OMAR A. HEWEDY *, A. M. EL-ZANATY, A. I. FAHMI

Department of Genetics, Faculty of Agriculture, Menoufia University, Shibin, El-Kom, Egypt

Abstract

The present study aimed to isolate indigenous *Trichoderma* fungi possessing cellulolytic activities from different geographical locations in Egypt. Such strains can be used to convert crop residues to bioproducts, which presently is one of the most important issues in developing countries. The isolates were identified using molecular approaches and morphological characteristics. Favorable growth conditions for *Trichoderma* isolates (media & temperature) were assessed *in vitro*. The growth rate of *Trichoderma* isolates was assessed on two media, namely Potato Dextrose Agar (PDA) and Synthetic Nutrient Agar (SNA), and morphological parameters, i.e., shape, pigment, and edge were observed. A comparison of sequences encoding for internal transcribed spacer (ITS) and translation elongation factor 1-α (*tef1*) was conducted to determine the species. Cellulase assay was performed using different cellulose substrates (Swollen, CMC (low viscosity carboxymethylcellulose), and Avicel). Among the isolated fungi, *Trichoderma viride* EGY-T27 had the highest endoglucanase activity, while *Trichoderma harzianum* EGY-T30 showed the highest exoglucanase activity. A total of 27 strains of *Trichoderma* were identified using ITS, and the *tef1* gene was sequenced for 23 strains. The tested strains were identified with acceptable quality based on ITS sequences, which ranged from 98 to 100%, except for strain MH908499 (85%). This research constitutes the most comprehensive study on screening for fungal strains with the highest biodegradable potential isolated from various locations in Egypt.

Key words: *Trichoderma*, cellulase activity, biodegradation, ITS, *tef1*

Introduction

Trichoderma species are well known for their ability to colonize different substrates and diverse environmental habitats. They inhabit the rhizosphere layer of the soil with a rapidly growing rate, producing a large number of spores and degrading enzymes for their nutrition. Additionally, *Trichoderma* fungi are considered to be one of the most widely distributed fungi in nature and can grow in a wide range of climates (Seidl, 2006; Topolovec-Pintarić, 2019). Some *Trichoderma* strains are endophytes that promote plant health and serve as biocontrol agents via mycoparasitism; they are also used as biopesticides instead of synthetic pesticides and as biofertilizers for enhancing root development (Arnold et al., 2003; Benítez et al., 2004; Harman et al., 2004; Barari, 2016). *Trichoderma* can also grow on various substrates such as wood, soil, crop residues, and some plant habitat niches as endophytes (Samuels et al., 2006; Bailey and Melnick, 2013; Chaverri and Samuels, 2013). They are currently widely used in industrial applications because of their ability to produce extracellular hydrolases in large amounts (Mach and Zeilinger, 2003; Samuels et al., 2006). Their ability to produce numerous digestive enzymes such as cellulose, hemicellulose, lignin, and chitin that break down various biopolymers have been utilized as a strategy for degrading various agricultural wastes and biomass, with biofuel as the most valuable product of this hydrolysis process (Druzhinina et al., 2018c). The development of alternative energy strategies is a critical factor because of the rising prices of crude oil as well as for reducing environmental pollution. Therefore, bioconversion of lignocellulosic residues

* Corresponding author: Department of Genetics, Faculty of Agriculture, Menoufia University, Shibin, El-Kom, Egypt; e-mail: hewedy.omar@gmail.com

is initiated primarily by microorganisms such as *Trichoderma reesei* and *Aspergillus niger*. *T. reesei* is a *Trichoderma* species that is commercially used for the production of cellulolytic enzymes required for making biofuels and is also used in other industries (Dashtban et al., 2009; Druzhinina et al., 2018c).

Cellulose and hemicellulose polysaccharides are β-(1,4)-linked glucose polymers with diverse compositions. Evidently, they constitute 60–80% of the plant cell wall. This lignocellulosic biomass, which includes forest wastes and residues of crops, is considered the world's most abundant natural biopolymer and renewable resource on the Earth (Singhania et al., 2013). It is a promising sustainable source of substrate in the production of bioethanol (Gusakov, 2013). Nevertheless, the high price of cellulase extraction and processing increases the cost of biofuel production compared to that of fossil fuels, which limits the commercial production of biofuel on a large scale (Wu et al., 2011). A promising strategy for efficient utilization of renewable resources is the hydrolysis of lignocellulosic biomass by filamentous fungi such as *Trichoderma*. Cellulase enzymes comprise a mixture of three major enzyme classes: endoglucanase (EC 3.2.1.4) that attack multiple internal sites in the regions of the cellulose fiber, cellobiohydrolase (EC 3.2.1.91), and glucosidase (EC 3.2.1.21) (El-Zawahry et al., 2010; Adsul et al., 2019). These enzymes are used not only in the biodegradation of wastes, but they also have extensive applications in animal feed; textiles; grain alcohol fermentation; pulp and paper processing; starch processing; and pharmaceutical, malting, and food industries (Gordillo-Fuenzalida et al., 2019; Zhanga et al., 2019). *Trichoderma* can produce high yields of endo/ exoglucanases, but its low yield of β-glucosidase has diminished its industrial value (Dashtban and Qin, 2012; Tiwari et al., 2013; de Souza et al., 2018). By using a series of chromatographic procedures, four endoglucanases (Endo I, II, III, and IV) and one exoglucanase (Exo II) were isolated from a commercial cellulase preparation derived from *T. viride* (Kim et al., 1994). Additionally, *T. reesei* is considered as one of the most industrially useful *Trichoderma* species for cellulase processing (Mandels and Reese, 1957; Gomes et al., 1992; Keshavarz and Khalesi, 2016; Druzhinina and Kubicek, 2017b).

Glucose is the most abundant monosaccharide produced in plant tissues and most algae during photosynthesis. Therefore, the conversion of cellulosic and hemicellulosic polymers into bioethanol *via* glucose or platform chemicals is considered as an important alternative method to reduce carbon dioxide emission and reduce the overdependence on fossil fuels (Schuster et al., 2012). The traditional methods used for eliminating cellulolytic materials and biological wastes have a negative influence on the environment and climate. Moreover, global energy demand is increasing with the rapidly growing human population, and recently, the rapid consumption of fossil fuels, for instance, has resulted in its depletion, which has raised questions concerning environment safety. Lignocellulosic biomass has emerged as an alternative solution for these issues because of the high cost of cellulase preparation and difficult fermentation process and to reduce the destructive effect on environment due to the burning of rice straw for instance. It is therefore necessary to enhance the hydrolytic efficiency of promising strains such as *Trichoderma* for lignocellulosic biomass.

The present study evaluated the diversity of *Trichoderma* isolates in several Egyptian locations. Thus, the present work focused on the identification of representative Egyptian *Trichoderma* isolates, determination of their favorable growth conditions, and screening of the cellulase activity of fungi from native environmental sources at different geographical locations to select the promising isolates of cellulase production.

Materials and methods

Fungal isolates and collection sources

A library of 40 *Trichoderma* isolates was randomly collected from the rhizosphere soil samples associated with various crop residues located at 38 different geographic habitats in Egypt (Table 1, Fig. 1). Each sample contained 200 g of soil collected from a depth of approximately 20 cm. The samples were placed in sterile polyethylene bags, transported to the laboratory, and stored at 4 °C until isolation according to the method of Elad et al. (1981). Briefly, approximately 10 g of each homogeneous soil sample was weighed and transferred to a 250 ml Erlenmeyer flask containing 100 ml of sterile water and shaken at 150 rpm for 2 h to make the stock solution. Subsequently, a series of 10-fold dilutions of the suspension were prepared for each sample, and 100 µl of each dilution was plated on Potato Dextrose Agar (PDA). The isolates of *Trichoderma* spp. were sub-

Table 1. Different crop residues for *Trichoderma* isolation collected from different geographical locations

Isolate code	Habitats	Crop residues	Soil type	No. of location(s) surveyed	No. of *Trichoderma* isolates
EGY-T1	Quwesna	corn	Peat	3	3
EGY-T2	Banha	rice straw	Clay Loam	4	4
EGY-T3	Berket-Elsaba	berssem	Loamy	2	1
EGY-T4	Tanta	rice straw	Clay Loam	3	1
EGY-T5	Kafer-Elshikh	rice straw	Clay Loam	3	1
EGY-T6	Behra	tomato	Loamy	5	1
EGY-T7	Alexandria	rice straw	Clay	1	1
EGY-T8	Menia	tomato	Loamy	3	1
EGY-T9	Cairo	grape	Sandy – Clay	3	0
EGY-T10	Alarish	olive	Calcareous	1	0
EGY-T11	North-Sinai	pomegranate	Calcareous	1	1
EGY-T12	Elein- Elsokhna	pomegranate	Sandy	2	1
EGY-T13	Suiez	citrus	Chalk	3	1
EGY-T14	Assiut	sugar cane	Silt	2	1
EGY-T15	Sallum	barley	Calcareous	1	0
EGY-T16	Giza	eggplant	Sandy – Clay	1	0
EGY-T17	Fayum	cotton waste	Clay Loam	3	1
EGY-T18	Helwan	corn	Sandy	4	1
EGY-T19	Giza	tomato	Sandy – Clay	2	0
EGY-T20	Santkatrein	olive	Calcareous	4	2
EGY-T21	Damietta	rice straw	Loamy	3	1
EGY-T22	Zagazig	wheat	Clay Loam	3	0
EGY-T23	Sadat	grape	Limestone saline	4	1
EGY-T24	Zefta	cotton waste	Sandy – Clay	3	3
EGY-T25	Fayed	citrus	Sandy	4	2
EGY-T26	Dakahlia	rice straw	Clay Loam	2	0
EGY-T27	Rafah	olive	Calcareous	2	1
EGY-T28	Beni Suef	onion	Calcareous	3	1
EGY-T29	Taba	cantaloupe	Sandy	1	0
EGY-T30	Sohag	onion	Sandy	3	1
EGY-T31	Mansoura	rice straw	Loamy	3	2
EGY-T32	New Valley	onion	Loamy	2	0
EGY-T33	Assiut	cotton waste	Clay	2	0
EGY-T34	Ismailia	corn	Sandy	3	0
EGY-T35	New Nubariya	mango	Sandy – Clay	3	0
EGY-T36	Luxor	peach	Sandy – Clay	3	2
EGY-T37	Beni Suef	olive	Sandy	1	0
EGY-T38	Qena	onion	Sandy – Clay	2	1
EGY-T39	Aswan	barley	Clay Loam	2	0
EGY-T40	South Sinai	bean	Calcareous	2	2

Fig. 1. Examples of different crop residues from different geographical locations used for *Trichoderma* isolation

cultured and purified by the spread plating technique to obtain monosporic cultures (Rodrigues et al., 2014). All the studied isolates were cultured on PDA containing 200 g potato infusion, 20 g dextrose, and 20 g agar (per/l) supplemented with streptomycin sulfate (0.4 mg/ml) in 9 cm Petri plates and incubated at 28°C for 5–7 days. *Trichoderma* cultures were maintained on PDA slants and stored in 50% glycerol stocks at –20°C for future studies.

Morphological characterization of Trichoderma spp.

Trichoderma spp. were identified on the basis of their morphological and cultural characteristics, which included several morphological parameters such as colony shape, color, and edge and the growth rate of *Trichoderma* fungi on different culture media. Growth rates of pure *Trichoderma* cultures and the optimum temperature for growth were determined on two different media, namely Potato Dextrose Agar (PDA) following the protocol of Samuels et al. (2002) and Synthetic Nutrient Agar (SNA) according to Nirenberg (1976), in 9 cm Petri plates at 25°C and 30°C. The colony radius was measured each day during the incubation period at intervals of 24 h until 120 h at 25°C and 30°C, with 12 h light and 12 h darkness. To prepare inoculum, the cultures were incubated at 28°C for 5 days on PDA, and the inoculum was placed at 5–10 mm distance from the edge of the plate by using an agar disc (5 mm diameter) with a five-day-old *Trichoderma* mycelium. Additional characteristics of *Trichoderma* colonies were noted, including growth rate, the shape of colonies, colony edge, and the occurrence of pigment diffusion in the agar. The color of each isolate was noted by inverting Petri dishes to observe the different diffusing pigments between the isolates. Each growth rate experiment was repeated three times, and the results were averaged for each isolate.

Molecular characterization of Trichoderma spp.

DNA Extraction and PCR Amplification

Genomic DNA was isolated from the cultivated fungal mycelium in 20 ml of Potato Dextrose Broth (PDB) at 28°C on a rotary shaker (200 rpm) for 5 days. The mycelium was harvested by filtration through mesh sieves (40 μm), washed with sterile water, and deposited on a Whatman filter paper (Sigma-Aldrich, India) to remove excess water. The mycelium was then ground to a fine powder in liquid nitrogen by using a sterilized mortar, and DNA was extracted according to the manufacturer's instructions by using Norgen's Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Canada) with some modifications of the protocol by (Dodd et al., 2002; Chi et al., 2009).

The DNA samples were purified using the spin column method of the DNA isolation kit and stored at –20°C for PCR amplification. The quality of DNA was assessed in 1% agarose gel, which was run in an horizontal electrophoresis unit (Bio-Rad, USA) at 100 V for

30 min. The image of DNA was captured by the E-Gel™ Imager System with E-Gel™ Adaptor. The sequences of fungal internal transcribed spacer (5.8SITS) amplified using a pair of general primers ITS4 (5′–TCCTCCGCT TATTGATATGC–3′) and ITS5 (5′–GGAAGTAAAAGT CGTAACAAGG–3′) (Eurofins) as described by (Möller et al., 1992; Schoch et al., 2012; Montoya et al., 2016). The other amplified gene coded for transcription elongation factor 1-α (*tef1*). PCR amplification was performed using the *tef1* primers (Eurofins): EF1-728F: (5′–CATC GAGAAGTTCGAGAAGG–3′) and TEF1R: (5′–GCCATC CTTGGAGATACCAGC–3′) as reported by (Samuels et al., 2002). PCR amplification of the ITS and *tef1* was performed in a total volume of 20 µl reaction mixture containing 0.5 µl of each primer (10 pmol/µl), 1.0 µl of genomic DNA (10 ng/µl), 10 µl of 2 × PCR MasterMix buffer (Thermo Fisher Scientific Biosciences GmbH, Germany; 0.05 µg/µl Taq polymerase, 4 mM $MgCl_2$, and 0.4 mM dNTPs), and 8 µl of ultrapure sterile water using Thermal Cycler (BIO-RAD) according to (Montoya et al., 2016). After amplification, the PCR fragments were electrophoresed on 2% agarose gel with a 100 bp DNA ladder (MBI Fermentas) at 80 V for 20 min, stained with 0.5 µg/ml ethidium bromide water solution for 15 min, and examined by a gel imaging system (Bio-Rad, Gel Doc).

DNA Barcoding and phylogenetic analysis

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, USA). The purified PCR products were subjected to Sanger sequencing in binary directions with the BigDye terminator v3.1 sequencing kit and a 3730XL automated sequencer (Applied Biosystems, Foster City, CA) by using ITS4 and ITS5 to ensure the identification of *Trichoderma* isolates. Nucleotide sequences were determined on both strands of PCR amplification products at the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). The nucleotide sequences were assembled using Sequencer ABI PRISM 3730XL Analyzer. FASTA sequence alignments were conducted with BLASTN programs and compared with the reference sequences deposited in the NCBI GenBank database. Phylogenetic tree affiliations were generated by a Neighbor-Joining method using MEGA version 7.0 (Kumar et al., 2016). The 5.8S-ITS sequences were compared to a specific database for *Trichoderma* by using the TrichOKEY2 program http://www.isth.info/ (Druzhinina et al., 2005a). The GenBank accession numbers for the sequences included in the analysis are listed in Table 3. Phylogenetic data were obtained by aligning the nucleotides of both ITS rDNA and tef1 retrieved from the BLAST algorithm (Fig. 3).

Evaluation of the potential of Trichoderma isolates to produce cellulases

The collection of 40 *Trichoderma* isolates was screened for their ability to produce cellulase and to determine cellulase activity by using different substrates such as Swollen, Avicel, and carboxymethyl cellulose (CMC) following a protocol by Fahmi et al. (2016). The experiments were performed in triplicate for statistical analysis.

Statistical analysis

All statistical analyses were performed by one-way analysis of variance (ANOVA) using IBM SPSS Statistics v25.0 HF001 IF007 software.

Results

Habitat and distribution

Forty fungal isolates were collected from soil samples associated with 20 types of crop residues, including corn, tomato, wheat, grape, olive, and onion, from 38 geographic habitats in Egypt as shown in Table 1 and Figure 1. The cultures of each isolate were maintained for further experimentation. Five replicates were obtained from each geographic site as a representative of various Egyptian locations to study the biodiversity of the isolated fungi based on the cultivated crop and climatic conditions.

Sample collection and Trichoderma isolation

Egypt's agricultural production, food security, and environmental conservation depend on the country's remarkable biodiversity. Egypt's agrobiodiversity encompasses not just its wide variety of species and genetic resources but also the numerous practices that farmers employ to use, enhance, and conserve this diversity (Lloyd, 2017). Rhizosphere soil samples were screened for *Trichoderma* genera with the highest cellulase activities. Forty *Trichoderma* isolates were successfully isolated from the rhizosphere soil layer of 19 different crop residues (Table 1). The samples of different rhizosphere soils were examined to determine the soil type of each

location at the Soil, Water and Environment Research Institute in Egypt, which are summarized in 10 soil types as shown in Table 1.

Temperature effect on the growth rate of Trichoderma isolates in vitro

The growth rates of *Trichoderma* isolates were measured after 1, 2, 3, 4, and 5 days at two different temperatures (25°C and 30°C) while inoculated on two different growth media (PDA and SNA). The results (Table 2, Fig. 2) showed a significant variation in the culture growth depending on the type of media used and incubation temperature. Significant differences were observed in a community of *Trichoderma* species from soils with different crop types. Additionally, the sporulation patterns of the isolated *Trichoderma* species were affected by media type and temperature. The growth rates varied notably between the studied isolates, for instance, *Trichoderma* EGY-T3 from loamy soil and berssem residues showed the highest growth rate of 6.36 and 6.10 cm after 5 days of incubation on PDA media at 25°C and 30°C, respectively. *Trichoderma* isolates EGY-T10 and EGY-T15 had the highest growth rate ranging between 4.26 and 4.10 cm on PDA and SNA media at 25°C and 30°C, respectively. On the other hand, the isolated fungal strains EGY-T40 and EGY-T32 from South Sinai and New Valley had the lowest growth rates (3.38 and 3.90 cm, respectively) at 25°C and 30°C on PDA. These isolates were collected from bean and onion residues from calcareous and loamy soils. The inoculation of *Trichoderma* EGY-T12 and EGY-T10 isolates on SNA at 25°C and 30°C showed the lowest growth rates of 2.08 and 1.24 cm, respectively, after 5 days from inoculation. These results indicate that the fungal strain, nutrient source, and temperature influence the growth rates of the studied *Trichoderma* isolates (Fig. 2A, 2B and 2C). The second parameter used for morphological observations was the color of the backside of Petri dishes, which is a morphological characteristic to differentiate the studied isolates (Fig. 2D). Although the morphological identification clearly indicated that the fungal isolates were *Trichoderma,* its ability to classify them into species was not quite precise. Therefore, molecular identification was used as a second method to clearly classify the fungal isolates.

DNA sequencing and phylogenetic analysis of ITS and tef1 genes

To identify *Trichoderma* species, several molecular methods were conducted. The sequences of ITS and *tef1* genes were sequenced to identify the strains and study the biodiversity relationship between them. The sequences of the ITS fragments (ITS4–ITS5) were analyzed using the nucleotide BLASTN program. The National Center for Biotechnology Information (NCBI) database was used to assess the similarity by comparing to homologous sequences deposited in NCBI GenBank https://www.ncbi.nlm.nih.gov/nuccore.

One band of 620 bp was amplified using ITS primers, and one band of 610 bp was obtained in the amplification of the *tef1* gene. Purified PCR products were sequenced, and the data were compared with the published ITS data on NCBI by using BLASTn analysis. The identified *Trichoderma* species are summarized in Table 3 along with their corresponding NCBI accession numbers (MH908491––MH908517). A total of 7 strains of *T. harzianum* Clade, 7 strains of *T. afroharzianum* Clade, 5 strains of *T. asperellum* Clade, 3 new species, and one of each taxa with *T. asperelloides*, *T. aureoviride, T. longibrachiatum*, *T. viride,* and *T. atrobrunneum* strains were detected. ITS sequences provide poor resolution for differentiation of closely related species (Case et al., 2007); moreover, the phylogenetic analysis positioned three unknown species in *Trichoderma* fungi. Hence, for more detailed determination of fungal species, the amplification of *tef1* was used for comparing both similarities and differences between the isolates to confirm that the identified species was *Trichoderma*. In addition, there are varying relative frequencies of occurrence of isolates in the different locations with different crop residues in soils associated with different crop cultivation. Interestingly, the most relative frequency of occurrence of the isolates was *T. harzianum* under different seasonal crops such as tomato, onion, and rice crops in loamy soils. Furthermore, *T. harzianum* from loamy soils was more common than that in sandy soils. Furthermore, only *T. viride* was isolated from olive crop residues in calcareous soils, while *T. atrobrunneum* and *T. asperelloides* were isolated from bean and corn residues, respectively. The distribution of four nucleotides (ATGC) and the length of the ITS region of the identified strains are given in Table 4.

Table 2. Colony characteristics of different *Trichoderma* isolates grown on PDA after seven days of incubation in dark at 28°C and the average of growth rate per day cultured on PDA and SNA in dark for five days at 25°C and 30°C

Isolate code	Colony			Growth rate			
	shape	reverse color	edge	PDA		SNA	
				25°C	30°C	25°C	30°C
EGY-T1	circular	green	smooth	5.24 fghij	4.90 hijklmnop	3.76 bcdefg	2.86 jkl
EGY-T2	serrate	white	wavy	5.00 hij	4.64 mnopqr	2.44 mnopq	2.38 m
EGY-T3	circular	yellow	smooth	6.36 a	6.10 a	3.74 bcdefg	3.90 ab
EGY-T4	circular	light green	smooth	5.72 cdef	5.16	3.49 mnopq	3.00 hij
EGY-T5	circular	yellow	smooth	5.98 abc	5.58 ab	3.36 efghij	3.34 defgh
EGY-T6	circular	dark green	smooth	4.90 j	5.26 cdefgh	3.20 ghijkl	3.82 abc
EGY-T7	circular	white	smooth	5.38 cdefghij	4.96 ghijklmno	4.12 abcd	3.08 ghij
EGY-T8	serrate	white	wavy	5.30 defghij	4.60 opqr	2.58 lmnopq	1.74 nop
EGY-T9	circular	yellow	wavy	5.42 bcdefghij	4.30 r	2.94 ijklmn	3.60 bcde
EGY-T10	serrate	white	smooth	5.52 cdefghij	3.94 s	4.26 ab	1.24 q
EGY-T11	circular	green	smooth	5.48 cdefghij	5.00 efghijklm	2.62 lmnopq	1.60 op
EGY-T12	serrate	white	wavy	5.32 defghij	4.84 ijklmnop	2.08 q	1.96 n
EGY-T13	circular	yellow	wavy	5.70 cdef	5.66 b	3.62 cdefgh	3.60 bcde
EGY-T14	circular	light green	smooth	5.64 cdefgh	5.16 defghijk	2.64 lmnopq	3.56 bcde
EGY-T15	circular	light green	smooth	5.44 bcdefghij	5.26 cdefgh	3.88 abcdef	4.10 a
EGY-T16	serrate	light yellow	wavy	5.48 cdefghij	4.70 mnopq	2.84 jklmnop	2.34 m
EGY-T17	serrate	off-white	smooth	5.30 efghij	4.74 lmnop	2.50 mnopq	1.86 no
EGY-T18	circular	dark green	smooth	5.60 cdefgh	5.34 bcdef	3.28 fghijk	3.50 cdef
EGY-T19	cylindrical	light green	smooth	5.04 ghij	4.58 pqr	3.86 bcdef	2.86 jkl
EGY-T20	circular	light green	wavy	5.48 cdefghij	4.98 fghijklmn	3.54 defghi	2.70 kl
EGY-T21	circular	light green	smooth	5.38 cdefghij	4.72 mnopq	3.78 bcdefg	3.56 bcde
EGY-T22	circular	dark yellow	smooth	5.80 abcde	4.92 hijklmnop	3.76 bcdefg	4.10 a
EGY-T23	serrate	white	wavy	4.94 ij	4.36 qr	3.76 bcdefg	3.20 fghi
EGY-T24	cylindrical	dark green	smooth	5.64 cdefgh	4.68 mnopq	3.44 efghij	3.66 bcd
EGY-T25	circular	dark green	smooth	5.60 cdefgh	5.18 defghij	3.04 hijklm	3.50 cdef
EGY-T26	circular	light green	smooth	5.22 fghij	5.30 bcdefg	2.24 pq	3.56 bcde
EGY-T27	serrate	yellow	wavy	4.88 j	4.38 qr	2.68 klmnopq	2.94 ijk
EGY-T28	serrate	dark green	wavy	5.64 cdefgh	4.76 lmnop	2.70 klmnopq	3.26 efghi
EGY-T29	serrate	yellow	wavy	5.38 cdefghij	4.36 qr	3.94 abcde	2.86 jkl
EGY-T30	serrate	dark yellow	wavy	4.94 ij	4.62 nopqr	3.62 cdefgh	2.54 lm
EGY-T31	circular	yellow	smooth	5.50 cdefghij	5.20 defghi	2.92 ijklmno	3.34 defgh
EGY-T32	serrate	light green	smooth	5.60 cdefghi	3.90 s	4.20 abc	3.20 fghi
EGY-T33	circular	yellow	wavy	6.02 ab	5.50 abc	2.84 jklmnop	3.90 ab
EGY-T34	circular	light green	smooth	5.52 cdefghij	5.48 bcd	2.90 jklmno	3.90 ab
EGY-T35	cylindrical	light green	smooth	5.98 abc	5.10 efghijkl	2.56 mnopq	1.46 pq
EGY-T36	circular	yellow	smooth	5.92 abcd	5.60 ab	3.58 defgh	3.90 ab
EGY-T37	cylindrical	light green	smooth	4.86 j	4.84 ijklmnop	3.30 opq	3.60 bcde
EGY-T38	circular	yellow	smooth	5.84 abcde	5.56 ab	3.20 ghijkl	3.40 defg
EGY-T39	circular	white	smooth	4.04 k	4.80 klmnop	4.46 a	3.60 bcde
EGY-T40	cylindrical	green	wavy	3.38 l	4.82 jklmnop	2.32 nopq	1.66 nop

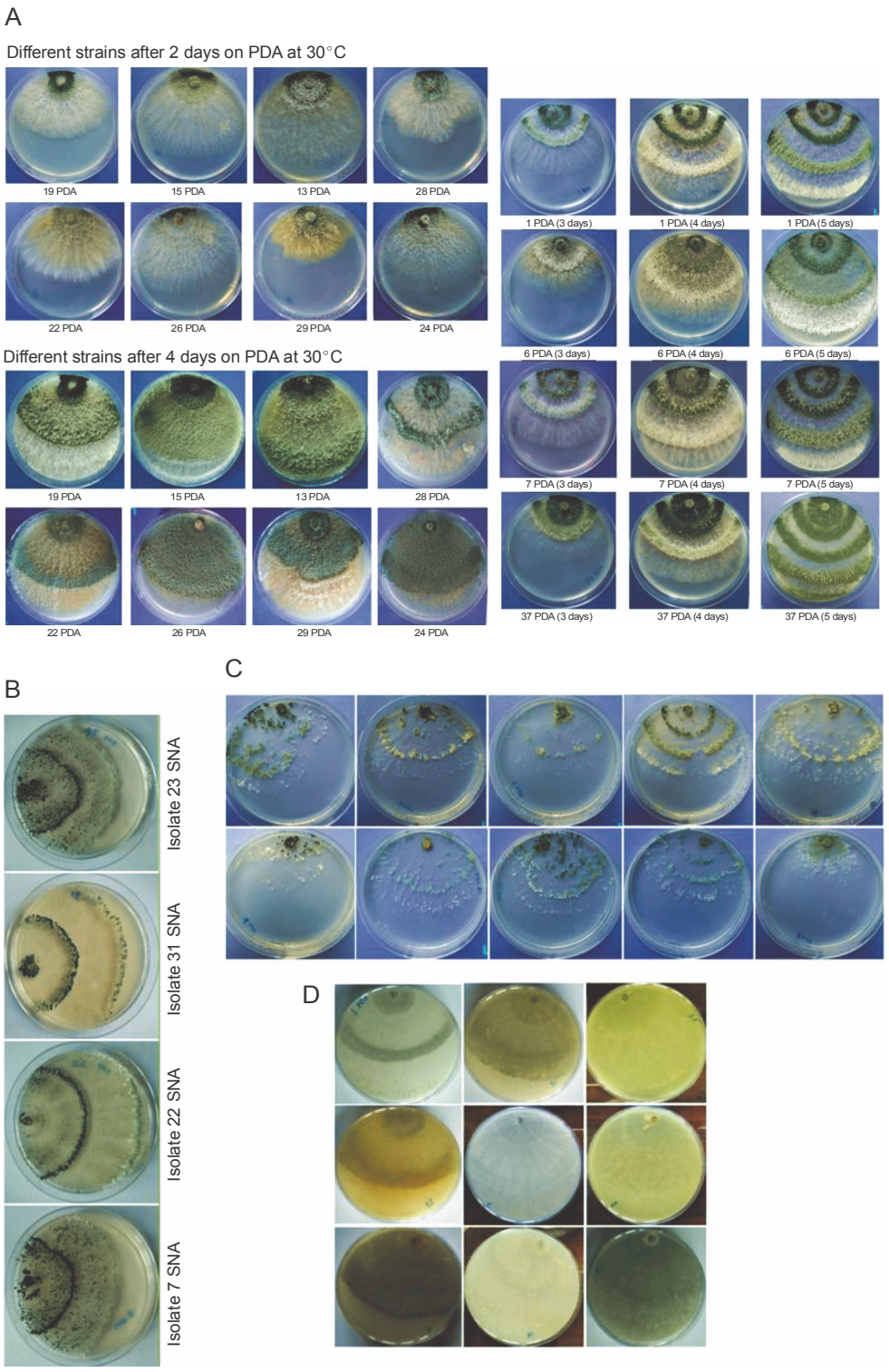

Fig. 2. Morphological characteristics of different *Trichoderma* isolates on PDA and SNA growth media to illustrate the growth rate and reverse color of each *Trichoderma* isolate: A) different fungal cultures on PDA after 2, 3, 4, and 5 days of growth, incubated at 30°C, B and C) cultures of different *Trichoderma* isolates on SNA after 5 days of growth, incubated at 30°C and 25°C, respectively, D) different pigment colors of each *Trichoderma* fungi grown on PDA at 30°C (reverse plate)

Among the several molecular methods used to characterize fungal species, sequence analysis of the ITS region is the most commonly used molecular marker analysis (Dashtban and Qin, 2012). In eukaryotic cells, there are two ITSs flanking the 5.8S gene. The two spacers, together with the 5.8S gene, are normally referred to as the ITS region. The rRNA genes are universally conserved, while the ITS region and intergenic spacer (IGS) are highly variable (Lieckfeldt et al., 2002). Thus, the sequences of these regions can be effectively used for the identification of closely related species (White et al., 1990). The lengths of ITS4 and ITS5 ranged from 364 to 596 bp for *T. viride* (MH908510) and *T. longibrachiatum* (MH908502), respectively. In addition, EGY-T12, T36, and T38 (*T. afroharzianum*) and EGY-T23 and T28 (*Trichoderma spp.*) had the same size of ITS sequences of 576 bp (Table 4). Furthermore, EGY-T20 and T21 (579 bp) had the same size of ITS as EGY-T6 and EGY-T13 (575 bp); however, after sequencing analyses, they were identified as different species (*Trichoderma spp.*, *T. harzianum, T. aureoviride*). In general, the 27 identified isolates belonged to *Trichoderma*. Moreover, seven species were classified as *T. harzianum* and five as *T. asperellum*. The strains showed clear variations in the growth rates probably due to the diversity of strains and the collection sources from different geographic locations in Egypt. To determine the phylogenetic positions of the *Trichoderma* isolates, sequence alignments of 5.8S-ITS and *tef1* gene regions with the fungal strains were performed using the Basic Local Alignment Search Tool (BLAST). These fungal strains were identified on the basis of the NCBI taxonomy database using BLASTN search results (% identify and alignment length) within clades with acceptable quality for ITS sequences ranging from 98% to100%, except for *T. asperellum* (MH908499) – 85%; three *Trichoderma* species remained unidentified. The result of the phylogenetic analysis of ITS sequence data is given in Figure 3A. The maximum parsimony bootstrap value for ITS was 100, while for *tef1* (Fig. 3B), it ranged from 95% to 100%. The cluster of *Trichoderma* spp. based on both ITS and *tef1* data was divided into two principal groups. The nucleotide ITS sequences of the 27 strains are presented in Figure 3C. The results revealed that the sequence fragments of ITS and *tef1* were highly similar in *Trichoderma* species as shown in Table 3. The proportion and composition of *Trichoderma* species varied between soil samples taken from different provinces and depended on both crop residues and soil type.

Screening of Trichoderma isolates for cellulase activity

Forty isolates of *Trichoderma* were evaluated for endoglucanase activity and production of exoglucanase enzymes. Three cellulose substrates were used to test the cellulase activity of the studied strains, i.e., CMC with Congo red dye assessed by the formation of clear zone diameters; another assay was conducted for the determination of endonuclease activities by using the plate clearing assay of acid swollen cellulose (Swollen) as the substrate. Screening of highly producing exoglucanase isolates was performed using microcrystalline cellulose (Avicel) as the substrate. The isolates showed significant differences in cellulase activity as shown in Table 5 and Figure 4. *T. viride* (MH908510) had the highest endoglucanase activity after incubation for 7 days on both Swollen and CMC cellulose substrates, with a growth rate of 4.70 and 5.15 cm, respectively. *T. harzianum* (MH908512), *T. afroharzianum* (MH908513), and *T. asperellum* (MH908494) showed the highest exoglucanase activity on Avicel substrate observed as growth zones of 7.10, 6.35, and 6.35 cm, respectively. *T. asperellum* (MH908497) showed the lowest endoglucanase activity on Swollen substrate with the growth rate of 1.60 cm, and *T. afroharzianum* (MH908509) exhibited the lowest exoglucanase activity when inoculated on Avicel substrate with the growth rate of 2.80 cm.

Discussion

A total of 40 *Trichoderma* isolates were obtained from rhizosphere soil samples of cultivated fields from different locations in Egypt (Table 1). Identification, evaluation of optimum *in vitro* growth conditions, and cellulase production assessment were carried out in the present study to sift through *Trichoderma* isolates to find candidate strains with high cellulolytic activity. These strains can be beneficial for converting agricultural biomass to glucose, the essential substrate in the microbial production of bioethanol. *Trichoderma* isolates showed a distinct variation in their phenotypic characteristics in culture media along with apparent differences in cellulase activity. Sequence analysis of these strains revealed three new taxa in soil samples from different Egyptian habitats. It has been suggested that the diversity of soil sample locations might play a role in

Table 3. Isolate codes and GenBank accession numbers of *Trichoderma* strains using two molecular markers ITS and *tef1* sequences

Isolate code	NCBI accession no. ITS	Strains (ITS)	Strains (*tef* 1)
EGY-T1	MH908491	*Trichoderma asperelloides*	*Trichoderma asperelloides*
EGY-T2	MH908492	*Trichoderma asperellum*	*Trichoderma asperellum*
EGY-T3	MH908493	*Trichoderma harzianum*	*Trichoderma harzianum*
EGY-T4	MH908494	*Trichoderma asperellum*	*Trichoderma asperellum*
EGY-T5	MH908495	*Trichoderma harzianum*	*Trichoderma harzianum*
EGY-T6	MH908496	*Trichoderma harzianum*	*Trichoderma afroharzianum*
EGY-T7	MH908497	*Trichoderma asperellum*	*Trichoderma asperellum*
EGY-T8	MH908498	*Trichoderma harzianum*	*Trichoderma afroharzianum*
EGY-T9	–	–	–
EGY-T10	–	–	–
EGY-T11	MH908499	*Trichoderma asperellum*	
EGY-T12	MH908500	*Trichoderma afroharzianum*	*Trichoderma afroharzianum*
EGY-T13	MH908501	*Trichoderma aureoviride*	*Trichoderma sp.*
EGY-T14	MH908502	*Trichoderma longibrachiatum*	–
EGY-T15	–	–	–
EGY-T16	–	–	–
EGY-T17	MH908503	*Trichoderma asperellum*	–
EGY-T18	MH908504	*Trichoderma afroharzianum*	–
EGY-T19	–	–	–
EGY-T20	MH908505	*Trichoderma sp.*	*Trichoderma harzianum*
EGY-T21	MH908506	*Trichoderma harzianum*	*Trichoderma harzianum*
EGY-T22	–	–	–
EGY-T23	MH908507	*Trichoderma sp.*	*Trichoderma afroharzianum*
EGY-T24	MH908508	*Trichoderma afroharzianum*	*Trichoderma afroharzianum*
EGY-T25	MH908509	*Trichoderma afroharzianum*	*Trichoderma afroharzianum*
EGY-T26	–	–	–
EGY-T27	MH908510	*Trichoderma viride*	*Hypocrea lixii*
EGY-T28	MH908511	*Trichoderma sp.*	*Trichoderma afroharzianum*
EGY-T29	–	–	*Trichoderma afroharzianum*
EGY-T30	MH908512	*Trichoderma harzianum*	*Trichoderma harzianum*
EGY-T31	MH908513	*Trichoderma afroharzianum*	*Trichoderma afroharzianum*
EGY-T32	MH908514	*Trichoderma harzianum*	–
EGY-T33	–	–	–
EGY-T34	–	–	–
EGY-T35	–	–	–
EGY-T36	MH908515	*Trichoderma afroharzianum*	*Trichoderma afroharzianum*
EGY-T37			*Trichoderma afroharzianum*
EGY-T38	MH908516	*Trichoderma afroharzianum*	*Trichoderma afroharzianum*
EGY-T39	–	–	–
EGY-T40	MH908517	*Trichoderma atrobrunneum*	–

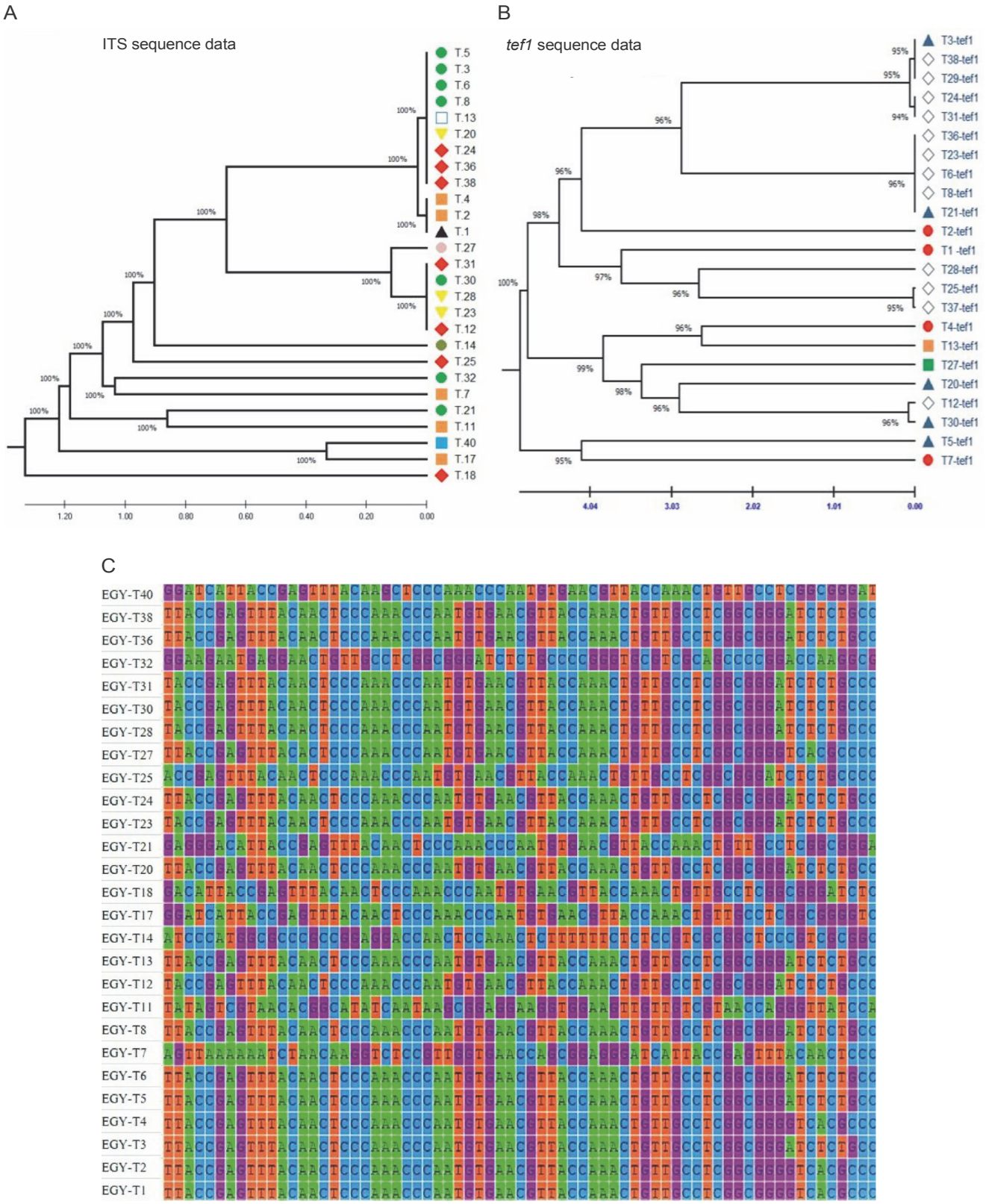

Fig. 3. Phylogenetic trees inferred from ITS rDNA and *tef1* sequences. A and B) neighbor-joining analysis of identified isolates using ITS and *tef1* sequence data sets individually by MEGA7 (each color on the phylogenetic tree means the same *Trichoderma* strain), C) the alignment sequences based on ITS identification of *Trichoderma* isolates

Table 4. ITS length and DNA/GC-content of the identified *Trichoderma* isolates

Isolate code	A [%]	T [%]	C [%]	G [%]	G+C [mol %]	ITS query length [bp]
EGY-T1	24.3	21.4	34.3	20	54.3	583
EGY-T2	24.3	21.4	34.3	20	54.3	571
EGY-T3	24.3	24.3	32.9	18.6	51.5	572
EGY-T4	24.3	21.4	34.3	20	54.3	560
EGY-T5	24.3	24.3	32.9	18.6	51.5	590
EGY-T6	24.3	24.3	32.9	18.6	51.5	575
EGY-T7	32.9	22.9	22.9	21.4	44.3	568
EGY-T8	24.3	24.3	32.9	18.6	51.5	593
EGY-T9	–	–	–	–	–	–
EGY-T10	–	–	–	–	–	–
EGY-T11	30	25.7	15.7	28.6	44.3	511
EGY-T12	24.3	22.9	34.3	18.6	52.9	576
EGY-T13	24.3	24.3	32.9	18.6	51.5	575
EGY-T14	12.9	22.9	41.4	22.9	64.3	364
EGY-T15	–	–	–	–	–	–
EGY-T16	–	–	–	–	–	–
EGY-T17	27.1	22.9	28.6	21.4	50	584
EGY-T18	27.1	24.3	30	18.6	48.6	577
EGY-T19	–	–	–	–	–	–
EGY-T20	24.3	24.3	32.9	18.6	51.5	579
EGY-T21	28.6	21.4	27.1	22.9	50	579
EGY-T22	–	–	–	–	–	–
EGY-T23	24.3	22.9	34.3	18.6	52.9	576
EGY-T24	24.3	24.3	32.9	18.6	51.5	594
EGY-T25	24.3	21.4	34.3	20	54.3	592
EGY-T26	–	–	–	–	–	–
EGY-T27	22.9	21.4	34.3	21.4	55.7	596
EGY-T28	24.3	22.9	34.3	18.6	52.9	576
EGY-T29	–	–	–	–	–	–
EGY-T30	24.3	22.9	34.3	18.6	52.9	574
EGY-T31	24.3	22.9	34.3	18.6	52.9	578
EGY-T32	17.1	14.3	31.4	37.1	68.5	546
EGY-T33	–	–	–	–	–	–
EGY-T34	–	–	–	–	–	–
EGY-T35	–	–	–	–	–	–
EGY-T36	24.3	24.3	32.9	18.6	51.5	576
EGY-T37	–	–	–	–	–	–
EGY-T38	24.3	24.3	32.9	18.6	51.5	576
EGY-T39	–	–	–	–	–	–
EGY-T40	27.1	22.9	28.6	21.4	50	582

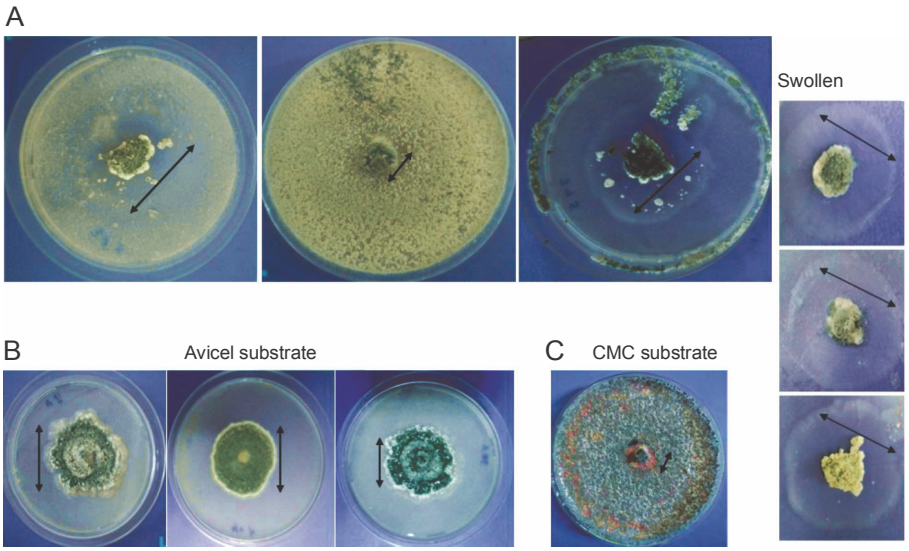

Fig. 4. Determination of *Trichoderma* isolates cellulase activity *in vitro* after 7 days of growth using different cellulose substrates: A) different growth zones of *Trichoderma* fungi using Swollen substrate, B) different growth rates of *Trichoderma* isolates on Avicel substrate, and C) *Trichoderma* EGY-T25 growth on CMC cellulose substrate

the variation of *Trichoderma* species (Rodrigues et al., 2014; Jiang et al., 2016). Because these strains were isolated from soil samples of different crop residues located at different regions in Egypt, the variation in cultivated crops and the isolation sources might also have an impact on the biodiversity of the fungal species. High level of *Trichoderma* biodiversity in the agricultural soil as observed in this study was based not only on morphological characteristics but also on molecular identification. The fungal diversity and the distribution pattern were influenced by different regions and crop types.

The cellulolytic activity of *Trichoderma* could be useful in the biofuel production process as it has been shown that *Trichoderma* could be used directly to hydrolyze cellulosic biomass to glucose, which is later fermented to produce bioethanol (Bu et al., 2019). Alternatively, these strains could act as a source for cellulases, thus reducing its production costs while increasing its sustainability (Libardi et al., 2017). In the present study, *Trichoderma* species were screened for the activity of cellulase enzymes using three cellulosic substrates: Swollen and CMC for endoglucanase and Avicel for exoglucanase. Libardi et al. (2017) reported that microcrystalline cellulose (Avicel) was the best inducer in terms of promoting high cellulase enzyme activity, and hence, it was used in the present study to assess cellulase activity as shown in Table 5 and Figure 4. Screening of cellulase-producing microorganisms can be performed on agar plates by using a cellulosic substrate such as Avicel or carboxymethylcellulose (CMC) as carbon source for microorganism growth. To identify isolates producing high levels of endoglucanases, two methods were performed: CMC and swollen cellulose plate assay. CMC is used as it resembles cellulose; Congo red colorizes cellulose, and the area that is decolorized indicates the endoglucanase enzyme activity (Florencio et al., 2012). *T. viride* (Rafah isolate) had the highest cellulolytic activity for swollen and CMC (4.70 and 5.15 cm, respectively), which is consistent with previous studies showing that *T. viride* has high cellulase activity (Penttilä et al., 1986; Fowler and Brown, 1992; Saloheimo et al., 2002). The use of Avicel revealed that *T. harzianum* (MH908512), *T. afroharzianum* (MH908513), and *T. asperellum* (MH908494) strains had the highest exoglucanase activity observed as 7.10, 6.35, and 6.35 cm, respectively. Although *Trichoderma* species are well known as efficient cellulose degraders and have been widely studied, this fungus produces a limited amount of β-glucosidase for a complete and efficient industrial cellulose hydrolysis (Sørensen et al., 2013).

Growth rate is a morphological parameter to assess morphological characteristics, while morphological characterization and molecular identification are essential

Table 5. Reported cellulase activity measurements of *Trichoderma* strains with PDA medium supplemented with different cellulose substrates after 7 days incubation at 28 ± 2°C

Isolate code	Endoglucanase		Exoglucanase
	Swollen	CMC	Avicel
EGY-T1	3.25 defgh	1.35 opq	3.60 ijkl
EGY-T2	3.00 efghijkl	1.25 pq	4.80 fgh
EGY-T3	2.60 hijklm	1.35 opq	4.90 fg
EGY-T4	2.25 lmn	1.75 mnop	6.35 bc
EGY-T5	3.70 bcde	1.00 q	3.60 ijkl
EGY-T6	3.05 efghijk	1.10 q	6.10 cd
EGY-T7	1.60 n	4.65 ab	5.25 ef
EGY-T8	2.95 efghijkl	1.80 lmnop	4.30 ghi
EGY-T9	3.35 cdefgh	1.40 opq	4.90 fg
EGY-T10	3.45 bcdef	1.90 klmno	3.90 ijk
EGY-T11	3.25 defgh	2.15 jklmn	3.75 ijkl
EGY-T12	2.35 jklm	1.10 q	3.35 klm
EGY-T13	3.15 defghi	1.40 opq	3.50 jkl
EGY-T14	3.50 bcdef	2.25 ijklm	4.10 hij
EGY-T15	2.85 fghijklm	1.40 opq	3.70 ijkl
EGY-T16	2.65 ghijklm	3.35 defg	5.25 ef
EGY-T17	3.10 defghij	1.55 nopq	5.10 ef
EGY-T18	2.85 fghijklm	1.60 nopq	4.10 hij
EGY-T19	2.85 fghijklm	3.75 cd	5.15 ef
EGY-T20	2.40 ijklm	2.75 ghij	3.25 klm
EGY-T21	3.40 bcdefg	2.45 hijk	4.75 fgh
EGY-T22	2.15 mn	4.25 bc	5.25 ef
EGY-T23	4.10 ab	1.10 q	4.90 fg
EGY-T24	2.30 klm	2.85 fghi	4.15 hij
EGY-T25	3.00 efghijkl	2.35 hijklm	2.80 m
EGY-T26	3.25 defgh	1.95 klmno	3.90 ijk
EGY-T27	4.70 a	5.15 a	5.65 de
EGY-T28	2.40 ijklm	2.40 hijkl	4.80 fgh
EGY-T29	2.40 ijklm	2.10 klmn	3.10 lm
EGY-T30	2.40 ijklm	2.30 hijklm	7.10 a
EGY-T31	3.10 defghij	3.75 cd	6.35 bc
EGY-T32	2.95 efghijkl	3.35 defg	5.10 ef
EGY-T33	3.10 defghij	3.45 def	5.25 ef
EGY-T34	4.60 a	4.40 b	6.75 ab
EGY-T35	3.35 cdefgh	4.35 b	6.10 bcd
EGY-T36	3.85 bcd	3.60 de	5.20 ef
EGY-T37	3.60 bcdef	2.90 fgh	3.85 ijk
EGY-T38	4.05 abc	3.10 efg	4.65 fgh
EGY-T39	3.35 cdefgh	2.85 fghi	5.15 ef
EGY-T40	3.10 defghij	1.90 klmno	4.10 hij

* 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

to confirm the taxonomy of the strains (Montoya et al., 2016). Based on the findings our study, the dominant species in the Egyptian soil samples were *T. harzianum* (7 strains) and *T. afroharzianum* (7 strains). Interestingly, the relationship between Harzianum clad dominance and their geographical locations might be due to both soil type and soil temperature. *T. harzianum* strains were isolated from loamy soils, and the optimum temperature for their growth ranged from 20 to 30°C. *T. harzianum* has worldwide distribution and is often the dominant species in many environments (Gherbawy et al., 2004; Sadfi-Zouaoui et al., 2009).

To identify unknown isolates, previous investigations on *Trichoderma* fungi used only classical taxonomic methods (morphological markers) or molecular markers using the sequence of the ITS region (Fisher et al., 1996; BARBOSA., 2004; Rodrigues et al., 2014). Morphological identification presents serious problems when used as the only tool for species identification in the genus *Trichoderma* (Chaverri et al., 2003; Druzhinina and Kubicek, 2005a). On the other hand, using only ITS as a taxonomical method of describing unknown *Trichoderma* isolates as a molecular tool can cause inaccurate identification with insufficient intraspecific variability (Atanasova et al., 2013; Chaverri et al., 2015). Hence, the combination of morphological and molecular characterization is more reliable for the classification of different strains (Taylor et al., 2000; Druzhinina and Kubicek, 2005a; Atanasova et al., 2013; Chaverri et al., 2015). Additionally, to enhance the accuracy of molecular classification, another molecular marker was used for sequencing these isolates: the *tef1* gene. These biodiverse isolates collected from different soils, crops, and locations are highly valuable for *Trichoderma* research through mapping the distribution of *Trichoderma* strains in Egyptian soils. The next step is further investigation to confirm the novelty of these taxa and field application with various agricultural wastes.

Conclusions

This research is the most comprehensive study to screen for biodegrading *Trichoderma* strains from several Egyptian locations and to study their potential in converting agricultural crop residues to bioproducts. This is one of the most important and pressing issues currently in developing countries and worldwide in general. Hence, the present study adds knowledge on the diversity of *Trichoderma* strains in Egypt. On the basis of taxonomic and cellulase activity of the isolated strains, the selection of the most promising strains is possible.

Author contributions

Omar Hewedy, the main author carried out all experiments, collected, assemble data, design the study, and wrote the manuscript. A. Fahmi developed the research concept and applied for the grant used to fund this work. A. El-Zanaty performed data analysis and interpretation. All authors read and approved the final manuscript.

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References

Adsul M., Sandhu S.K., Singhania R.R., Gupta R., Puri S.K., Mathur A. (2019) *Designing a cellulolytic enzyme cocktail for the efficient and economical conversion of lignocellulosic biomass to biofuels*. Enzyme Microb. Technol. 109442.

Arnold A.E., Mejía L.C., Kyllo D. (2003) *Fungal endophytes limit pathogen damage in a tropical tree*. Proc. Nat. Acad. Sci. 100: 15649–15654.

Atanasova L., Druzhinina I.S., Jaklitsch W.M., Mukherjee P., Horwitz B., Singh U. (2013) *Two hundred Trichoderma species recognized on the basis of molecular phylogeny. Trichoderma: biology and applications*. CABI, Wallingford: 10–42.

Bailey B.A., Melnick R.L. (2013) *The endophytic Trichoderma. Trichoderma: biology and applications*. CABI, Wallingford: 152-172.

Barari H. (2016) *Biocontrol of tomato Fusarium wilt by Trichoderma species under in vitro and in vivo conditions*. Cercetari Agron. Moldova 49: 91–98.

Barbosa V.S. (2004) *Efeito da fragmentaçao florestal na taxa de parasitismo de fungos associados ao jardim da formiga cortadeira Atta laevigata*. Universidade Federal de Pernambuco.

Benítez T., Rincón A.M., Limón M.C., Codon A.C. (2004) *Biocontrol mechanisms of Trichoderma strains*. Intern. Microbiol. 7: 249–260.

Bu Y., Alkotaini B., Salunke B.K., Deshmukh A.R., Saha P., Kim B.S. (2019) *Direct ethanol production from cellulose by consortium of Trichoderma reesei and Candida molischiana*. Green Proces. Synth. 8Ł 416–420.

Case R.J., Boucher Y., Dahllöf I., Holmström C., Doolittle W.F., Kjelleberg S. (2007) *Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies*. Appl. Environ. Microbiol. 73: 278–288.

Chaverri P., Branco-Rocha F., Jaklitsch W., Gazis R., Degenkolb T., Samuels G.J. (2015) *Systematics of the Tricho-*

derma harzianum species complex and the re-identification of commercial biocontrol strains. Mycologia 107: 558–590.

Chaverri P., Castlebury L.A., Overton B.E., Samuels G.J. (2003) *Hypocrea/Trichoderma: species with conidiophore elongations and green conidia*. Mycologia 95: 1100–1140.

Chaverri P., Samuels G.J. (2013) *Evolution of habitat preference and nutrition mode in a cosmopolitan fungal genus with evidence of interkingdom host jumps and major shifts in ecology*. Evolution 67: 2823–2837.

Chi M.-H., Park S.-Y., Lee Y.-H. (2009) *A quick and safe method for fungal DNA extraction*. Plant Pathol. J. 25: 108–111.

Dashtban M., Qin W. (2012) *Overexpression of an exotic thermotolerant β-glucosidase in Trichoderma reesei and its significant increase in cellulolytic activity and saccharification of barley straw*. Microbial Cell Fact. 11: 63.

Dashtban M., Schraft H., Qin W. (2009) *Fungal bioconversion of lignocellulosic residues; opportunities & perspectives*. Inter. J. Biol. Sci. 5: 578.

De Souza M.F., Da Silva A.S.A., Bon E.P. (2018) *A novel Trichoderma harzianum strain from the Amazon Forest with high cellulolytic capacity*. Biocatal.Agricult. Biotech. 14: 183–188.

Dodd S.L., Lieckfeldt E., Chaverri P., Overton B.E., Samuels G.J. (2002) *Taxonomy and phylogenetic relationships of two species of Hypocrea with Trichoderma anamorphs*. Mycol. Progr. 1: 409–428.

Druzhinina I., Kubicek C.P. (2005) *Species concepts and biodiversity in Trichoderma and Hypocrea: from aggregate species to species clusters?* J. Zhejiang Univ. Sci. B 6: 100.

Druzhinina I.S., Chenthamara K., Zhang J. (2018) *Massive lateral transfer of genes encoding plant cell wall-degrading enzymes to the mycoparasitic fungus Trichoderma from its plant-associated hosts*. PLoS Gen. 14: e1007322.

Druzhinina I.S., Kopchinskiy A.G., Komoń M., Bissett J., Szakacs G., Kubicek C.P. (2005) *An oligonucleotide barcode for species identification in Trichoderma and Hypocrea*. Fungal Gen. Biol. 42: 813–828.

Druzhinina I.S., Kubicek C.P. (2017) *Genetic engineering of Trichoderma reesei cellulases and their production*. Microbial Biotech. 10Ł 1485–1499.

El-Zawahry Y., El-Mougith A., El-Saadani M., Hafez E., Soliman S. (2010) *Partial purification and characterization of two endo-β-1, 4-glucanase from Trichoderma spp.(Shmosa tri)*. Aust. J. Basic Appl. Sci. 4: 4559–4568.

Elad Y., Chet I., Henis Y. (1981) *A selective medium for improving quantitative isolation of Trichoderma spp. from soil*. Phytoparasitica 9: 59–67.

Fahmi A., Eissa R., El-Halfawi K., Hamza H., Helwa M. (2016) *Identification of Trichoderma spp. by DNA barcode and screening for cellulolytic activity*. J. Microb. Biochem. Technol. 8: 202–209.

Fisher P., Stradling D., Sutton B., Petrini L. (1996) *Microfungi in the fungus gardens of the leaf-cutting ant Atta cephalotes: a preliminary study*. Mycol. Res. 100: 541–546.

Florencio C., Couri S., Farinas C.S. (2012) *Correlation between agar plate screening and solid-state fermentation for the prediction of cellulase production by Trichoderma strains*. Enzyme Res. doi: 10.1155/2012/793708

Fowler T., Brown R.D., (1992) *The bgI1 gene encoding extracellular β glucosidase from Trichoderma reesei is required for rapid induction of the cellulase complex*. Mol. Microbiol. 6: 3225–3235.

Gherbawy Y., Druzhinina I., Shaban G.M. (2004) *Trichoderma populations from alkaline agricultural soil in the Nile valley, Egypt, consist of only two species*. Mycol. Progr. 3: 211–218.

Gomes I., Gomes J., Steiner W., Esterbauer H. (1992) *Production of cellulase and xylanase by a wild strain of Trichoderma viride*. Appl. Microbiol. Biotech. 36: 701–707.

Gordillo-Fuenzalida F., Echeverria-Vega A., Cuadros-Orellana S., Faundez C., Kähne T., Morales-Vera R. (2019) *Cellulases production by a Trichoderma sp. using food manufacturing wastes*. Appl. Sci. 9: 4419.

Gusakov A.V. (2013) *Cellulases and hemicellulases in the 21st century race for cellulosic ethanol*. Taylor & Francis.

Harman G.E., Howell C.R., Viterbo A., Chet I., Lorito M. (2004) *Trichoderma species – opportunistic, avirulent plant symbionts*. Nature Rev. Microbiol. 2: 43.

Jiang Y., Wang J.L., Chen J. (2016) *Trichoderma biodiversity of agricultural fields in east China reveals a gradient distribution of species*. PLoS One 11.

Keshavarz B., Khalesi M. (2016) *Trichoderma reesei, a superior cellulase source for industrial applications*. Biofuels 7: 713–721.

Kim D.W., Jeong Y.K., Jang Y.H., Lee J.K. (1994) *Purification and characterization of endoglucanase and exoglucanase components from Trichoderma viride*. J. Ferment. Bioengin. 77: 363–369.

Kumar S., Stecher G., Tamura K. (2016) *MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets*. Mol. Biol. Evol. 33: 1870–1874.

Libardi N., Soccol C.R., Góes-Neto A., De Oliveira J., De Souza Vandenberghe L.P. (2017) *Domestic wastewater as substrate for cellulase production by Trichoderma harzianum*. Process. Biochem. 57: 190–199.

Lieckfeldt E., Kuhls K., Muthumeenakshi S. (2002) *Molecular taxonomy of Trichoderma and Gliocladium and their teleomorphs*. Trichoderma and Gliocladium Volume, 35.

Lloyd P. (2017) *Sustainable agriculture for biodiversity, biodiversity for sustainable agriculture*. Taylor & Francis.

Mach R., Zeilinger S. (2003) *Regulation of gene expression in industrial fungi: Trichoderma*. Appl. Microbiol. Biotech. 60: 515–522.

Mandels M., Reese E.T. (1957) *Induction of cellulase in Trichoderma viride as influenced by carbon sources and metals*. J. Bacteriol. 73: 269.

Mishra V.K., (2010) *In vitro antagonism of Trichoderma species against Pythium aphanidermatum*. J. Phytol. 2(9): 28–35.

Möller E., Bahnweg G., Sandermann H., Geiger H. (1992) *A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues*. Nucl. Acids Res. 20: 6115.

Montoya Q.V., Meirelles L.A., Chaverri P., Rodrigues A. (2016) *Unraveling Trichoderma species in the attine ant*

environment: description of three new taxa. Antonie van Leeuwenhoek 109: 633–651.

Nirenberg H. (1976) *Untersuchungen ber die morphologische und biologisch Diffrenzieerum in der Fusarium Sekion Lisiola*. Mitteil. Biol. Bundesanst. Fr. Land Forstwirtsch. 169: 1117.

Penttilä M., Lehtovaara P., Nevalainen H., Bhikhabhai R., Knowles J. (1986) *Homology between cellulase genes of Trichoderma reesei: complete nucleotide sequence of the endoglucanase I gene*. Gene 45: 253–263.

Rodrigues A., Passarini M.R., Ferro M. (2014) *Fungal communities in the garden chamber soils of leaf cutting ants*. J. Basic Microbiol. 54: 1186–1196.

Sadfi-Zouaoui N., Hannachi I., Rouaissi M. (2009) *Biodiversity of Trichoderma strains in Tunisia*. Canad. J. Microbiol. 55: 154–162.

Saloheimo M., Kuja-Panula J., Ylösmäki E., Ward M., Penttilä M. (2002) *Enzymatic properties and intracellular localization of the novel Trichoderma reesei β-glucosidase BGLII (Cel1A)*. Appl. Environ. Microbiol. 68: 4546–4553.

Samuels G.J., Dodd S.L., Gams W., Castlebury L.A., Petrini O. (2002) *Trichoderma species associated with the green mold epidemic of commercially grown Agaricus bisporus*. Mycologia 94: 146–170.

Samuels G.J., Dodd S.L., Lu B.S., Petrini O., Schroers H.J., Druzhinina I.S. (2006) *The Trichoderma koningii aggregate species*. Studies Mycol. 56: 67–133.

Schoch C.L., Seifert K.A., Huhndorf S. (2012) *Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi*. Proc. Nat. Acad. Sci. 109: 6241–6246.

Schuster A., Bruno K.S., Collett J.R. (2012) *A versatile toolkit for high throughput functional genomics with Trichoderma reesei*. Biotech. Biofuels 5: 1.

Seidl V. (2006) *Molecular and physiological investigations of biocontrol by the genus Hypocrea/Trichoderma*. Dissertation. Vienna University of Technology, Austria.

Singhania R.R., Patel A.K., Sukumaran R.K., Larroche C., Pandey A. (2013) *Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production*. Bioresource Tech. 127: 500–507.

Sørensen A., Lübeck M., Lübeck P.S., Ahring B.K. (2013) *Fungal beta-glucosidases: a bottleneck in industrial use of lignocellulosic materials*. Biomolecules 3: 612–631.

Taylor J.W., Jacobson D.J., Kroken S. (2000) *Phylogenetic species recognition and species concepts in fungi*. Fungal Gen. Biol. 31: 21–32.

Tiwari P., Misra B., Sangwan N.S. (2013) *β-Glucosidases from the fungus Trichoderma: an efficient cellulase machinery in biotechnological applications*. BioMed Res. Inter. doi: 10.1155/2013/203735

Topolovec-Pintarić S. (2019) *Trichoderma: invisible partner for visible impact on agriculture*. [in:] *Trichoderma – the most widely used fungicide*. IntechOpen.

White T.J., Bruns T., Lee S., Taylor J. (1990) *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics*. PCR protocols: a guide to methods and applications 18: 315–322.

Wood T.M. (1988) *Preparation of crystalline, amorphous, and dyed cellulase substrates*. Meth. Enzymol. 160: 19–25.

Wu G., He R., Jia W., Chao Y., Chen S. (2011) *Strain improvement and process optimization of Trichderma reesei Rut C30 for enhanced cellulase production*. Biofuels 2: 545–555.

Zhanga F., Bunterngsookc B., Lia J.X. (2019) *Regulation and production of lignocellulolytic enzymes from Trichoderma reesei for biofuels production*. Adv. Bioenergy 4: 79–119.