Egypt. J. Bot. **Vol. 63**, No. 3, pp. 981-1003 (2023)

Egyptian Journal of Botany
http://ejbo.journals.ekb.eg/

Morphological and Molecular Identification of Endophytic Fungi from Roots of Tomato and Evaluation of Their Antioxidant and Cytotoxic Activities

Samah A. El-Debaiky[(1)#], Ahmed F. El-Sayed[(2, 3)]
[(1)]Botany and Microbiology Department, Faculty of Science, Tanta University, Tanta, Egypt; [(2)]Microbial Genetics Department, Biotechnology Research Institute, National Research Centre, Giza, Egypt; [(3)]Egypt Center for Research and Regenerative Medicine (ECRRM), Cairo, Egypt.

ENDOPHYTIC fungi are identified as mitosporic and meiosporic ascomycetes that asymptomatically inhabit the internal tissues of some plants. They have a beneficial role in plant protection as more as producing several natural bioactive products. So, the present study aimed to isolate endophytic fungal species which associated with roots of tomato plants, sub-cultured in pure cultures and identified them according to their morphological and genetics characterization. The results introduced seven fungal species belong to the genera *Alternaria*, *Aspergillus*, *Penicillium*, *Talaromyces* and *Triangularia* which identified according to their cultural appearance and microscopic examination features. Their identification was confirmed by molecular characterization using sequencing the ITS and β-tubulin genes then deposited in GenBank with an accession numbers serial from OP599904.1 to OP599910.1 for ITS and from LC733668 to LC733674 for β-tubulin. Additionally, the antioxidant, antibacterial and antitumor studies of the crude filtrates of the isolated endophytic fungi revealed high antioxidant potential, negative antibacterial and successful antitumor activities.

Keywords: Endophytes, Genetic diversity, ITS, Morphological identification, β-tubulin, PCR.

Introduction

Endophytic fungi are defined as endosymbionts, mitosporic and meiosporic ascomycetes that colonize the internal healthy tissues of various plants for at least a part of their life cycles, asymptomatically via dormant infections (Sturz & Nowak, 2000; Wilson 2000). The endophytic fungi are found in about 300,000 different plants, including trees, vegetables, fodders, fruits, cereal grains, and other crops (Rosenblueth & Martínez-Romero, 2006 ; Younis, 2021; Sehim & Dawwam, 2022). Moreover, (Zabalgogeazcoa, 2008) stated that, endophytes have been found in all species of plants studied to date; however, the relationships among endophytes/plants are not well understood. Probably, some endophytes may increase plant growth and nutrient uptake, induce the plant's resistance to biotic and abiotic stresses, while some endophytic fungi may potentially become pathogens or saprophytes (Zabalgogeazcoa, 2008).

The genetically diverse endophytes have beneficial roles to their host plants where they produce bioactive compounds that have applied in many fields such as agriculture and pharmacology in producing the anticancer drugs (Strobel & Daisy, 2003; Govinda Rajulu et al., 2011; Chowdhary & Kaushik, 2015). Since few years researchers found that, endophytic fungi in medicinal plants can produce similar active substances to their host plants and various secondary metabolites (Jia et al., 2016).

Morphological examination of fungi, based on culture characters and microscopic features, is still central to mycology, and is the only method of identification of many fungi that have not yet been sequenced. Although, the identification of fungi morphologically is useful for the identification of

#Corresponding author email: samaheldebaiky@science.tanta.edu.eg Orcid: 0000-0002-5172-0222
Received 24/03/2023; Accepted 31/05/2023
DOI: 10.21608/ejbo.2023.201958.2291
Edited by: Prof. Dr. Khaled M. Ghanem, Faculty of Science, Alexandria University, Alexandira, Egypt.

the fungus up to the family or genus level (Wang et al., 2016) and is not enough to identify the isolate up to the species level (Lutzoni et al., 2004). Some fungal species including endophytes could not be identified by only morphological characteristics especially those which do not sporulate, so DNA sequence data were used for identification of these taxa (Guo et al., 2000, 2003).

The undifferentiated morphological appearance of medically significant fungus makes it difficult to identify them using traditional morphological criteria. In contrast, molecular techniques that use the polymerase chain reaction (PCR) to identify these fungi are quicker than phenotypic methods (Liu et al., 2000; Pryce et al., 2003). PCR amplification of the internal transcribed spacer region (ITS1- 5.8S-ITS2) is used to distinguish fungal species. Because of its conserved characteristic among species, the ITS region of nuclear ribosomal DNA has typically been regarded as a useful marker for molecular identification of fungus at the species level (Sánchez-Ballesteros et al., 2000). For example, the fungus *Aspergillus* was identified at species level and differentiated from other true pathogenic and opportunistic molds using ITS1 and ITS2, this process permeates the early diagnosis and screening of effective antifungal agents for patients (Henry et al., 2000). The beta-tubulin (β-tubulin) genes are found in all eukaryotes and have been used for phylogenetic analysis in fungi from kingdom to the species level. Reports have shown that β-tubulin genes have more variability compared to the ITS region. This amount of variation is suitable for determining phylogenetic relationship of closely related species of *Penicillium* and *Aspergillus* genera. Finally, a new pair of universal fungal primers; ITS1 and ITS4 were designed on the ITS region and used in distinguishing unambiguously the closely related species of both genera. (Visentin et al., 2009). The identification process was done by comparing the DNA sequence with those available from GenBank database.

Consequently, this research introduces isolation and identification of some endophytic fungi associated with roots of tomato plants by morphological and molecular characterization and studying their phylogenic relationship. Also, some the antioxidant, antibacterial and antitumor activities were carried out using the identified species.

Materials and Methods

Isolation and morphological identification of endophytic fungi

Tomato plants were cultivated in extremely hot weather during summer season 2020 where the temperature was about 40 ± 2°C. These plants suffered from blazing sun and high temperature which unsuitable for their growth. At the same time these tomato plants were subjected to a field experiment where they cultivated in infected soil with *Fusarium oxysporum* and treated by mint and clove oils. All these conditions were hindrance to normal growth of tomato plants.

Roots of these suffered plants were cut and washed thoroughly in running tap water for 5 min, for removing any clay or soil particles, and surface sterilized according to the method of Parthibhan et al. (2017). Sterilization was done by dipping roots in absolute ethanol for 30sec. and rinsed three times by sterile distilled water for 1 min. Each sterilized root sample was cut into small fragments (approximately 5mm). Then, the sterilized root segments were spread onto PDA plates containing 50 mg/l of the antibiotic chloramphenicol. The inoculated plates were incubated in dark at 30± 2°C for about 5-7 days. After the incubation period, cultures of the endophytic fungi that grew out from the roots were sub-cultured into other PDA plates to obtain pure cultures.

Plates of pure cultures were sealed with parafilm and maintained in the refrigerator at 4°C. Subsequently, for morphological characterization and microscopic examination, freshly sub-cultured pure cultures were clearly described and identified according to the identification keys cited in the identification books of fungi and literatures (Gilman, 1957; Ellis, 1971; Moubasher, 1993; Frisvad & Samson, 2004; Hubka et al., 2013).

Molecular characterization of endophytic fungi

DNA extraction

DNA extraction was performed using DNeasy Plant Mini Kit (Qiagen ,USA) according to the instructions of the manufacturer. The quality and quantity of DNA was analysed by gel electrophoresis.

Molecular identification

The molecular identification and confirmation of the isolated endophytic fungi was performed

using both ITS and β-tubulin gene regions separately. The ITS and the β-tubulin gene fragments were amplified using PCR technique using the DreamTaq kit (thermo scientific,USA) and according to the recommended protocol steps. Firstly, the ITS regions 1 & 2 of the ribosomal RNA (rRNA) gene complex, were amplified using the following primers: ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-CCTCCGC TTA TTGATATGC-3) (Table 1). The PCR process of ITS was carried out under the following conditions, 95°C for 3min, 35 cycles of 95°C for 0.5min, 52°C for 30sec, 72°C for 1min, and a final extension step of 72°C for 10min.

On the other hand, the β-tubulin gene region was amplified using the primers; bt2a (5-GGTAACCAAATCGGTGCTGCTTTC-3) and bt2b (5-ACCCTCAGTGTAGTGACCC-TTGGC-3) (Table 1) where the PCR conditions were: 95°C for 3min, 35 cycles of 95°C for 0.5min, 58°C for 30sec, 72°C for 1min, and a final extension step of 72°C for 10min. Then, all PCR products for both ITS and β-tubulin were separated by gel electrophoresis in 1.5% agarose and finally photographed using a gel documentation system.

Bioinformatics and phylogenetic analysis

ITS and β-tubulin amplicons were purified using gel extraction kit (Thermo fisher, USA) and directly sequenced in both directions using automatic DNA sequencer (Macrogen, Korea). Chromatograms were edited and trimmed using Bio Edit software. Edited sequences were aligned using Clustal X of ClustalW packages (Thompson et al., 1994) and submitted to GenBank, in the tree-based analysis, neighbor-joining (NJ) trees were constructed in MEGA x. Bootstrap support was estimated with 1000 heuristic replicates to test the reliability of inferred phylograms (Tamura et al., 2021). Then, according to nucleotide sequence, the ITS and β-tubulin sequences were submitted to the GenBank with accession numbers (OP599904-OP599910) for ITS and (LC733668-LC733674) for β-tubulin (Table 2).

Genetic diversity among isolated fungal strains using inter simple sequence repeat (ISSR) marker: ISSR-PCR

The genetic diversity among the tested endophytes was performed using six ISSR primers (Table 1). PCR process was conducted in a volume of 20 µL, consisted of 2µL of fungal DNA, 2µL of each ISSR primer separately, 10µL master mix PCR, and 6 µl double distilled (ddH_2O). PCR conditions included an initial denaturation for 5min at 94°C, followed by 45 cycles of 45sec at 94°C, 1min at 45°C, and 2min at 72°C with extension at 72°C for 10min. PCR products were separated by gel electrophoresis in 1.5% agarose and finally photographed using a gel documentation system (Grünig et al., 2001).

TABLE 1. Primers used for molecular identification of fungal isolates by PCR technique

Gene	Primer name	Direction	5` - OligoSeq - 3`	Target sequences	Annealing Temp.	References
ITS	ITS1	Forward	TCCGTAGGTGAACCTGCGG	600 bp	52°C	Visentin et al. (2009)
	ITS4	Reverse	CCTCCGCTTATTGATATGC			
β-tubulin	Bt2a	Forward	GGTAACCAAATCGGTGCTGCTTTC	550bp	58°C	
	Bt2b	Reverse	ACCCTCAGTGTAGTGACCCTTGGC			
ISSR	$ISSR_1$	One direction	ACACACACACACACACG	Variable	45°C	Grünig et al. (2001)
	$ISSR_2$	One direction	ACACACACACACACACT	Variable		
	$ISSR_3$	One direction	ACACACACACACACACC	Variable		
	$ISSR_4$	One direction	AGAGAGAGAGAGAGAGC	Variable		
	$ISSR_5$	One direction	CTCTCTCTCTCTCTCTG	Variable		
	$ISSR_6$	One direction	CTCTCTCTCTCTCTCTCTA	Variable		

TABLE 2. list of selected identified microorganisms with accession numbers

No	Code	Strain	Host	Accession number submitted to GenBank	
				ITS	β-tubulin
1	Egy1-EU1	*Aspergillus chevalieri* Egy1-EU1	Tomato Roots	OP599904.1	LC733668.1
2	Egy2-EU2	*Aspergillus chevalieri* Egy2-EU2	Tomato Roots	OP599905.1	LC733669.1
3	Egy3-NEO	*Talaromyces gossypii* Egy3-NEO	Tomato Roots	OP599906.1	LC733670.1
4	Egy4-YG	*Penicillium vanluykii* Egy4-YG	Tomato Roots	OP599907.1	LC733671.1
5	Egy5-SA1	*Penicillium capsulatum* Egy5-SA1	Tomato Roots	OP599908.1	LC733672.1
6	Egy6-SA5	*Alternaria arborescens* Egy5-SA5	Tomato Roots	OP599909.1	LC733673.1
7	Egy7-Gray	*Triangularia mangenotii* Egy7-Gray	Tomato Roots	OP599910.1	LC733674.1

ISSR data analysis

Genetic similarity based on the Jaccard coefficient was calculated using the SIMQUAL module and treed using the NTSYS version 2.20 software (Rohlf, 1998). According to the unweighted pair-group method using the arithmetic mean (UPGMA). Statistical cluster analysis and Principal Coordinate Analysis (PCoA) was performed to associate genetic relationships between species was performed with PAST 3.18 software (Hammer et al., 2001). The percentage polymorphism was calculated according to the following formula:

Polymorphism (%)= (number of polymorphic bands / total number of bands) × 100.

Evaluation of antioxidant activity of fungal extracts

ABTS radical scavenging activity

Each fungal culture previously grown on PDA broth medium was filtered using filter paper No.1 and two-fold dilutions (1, 0.5, 0.25mL/mL) were prepared from each filtrate using dist. H_2O to obtain total volume 1mL. The antioxidant activity of each fungal filtrate dilution against ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was tested according to the method of (Re et al., 1999). ABTS solution was diluted with methanol to an absorbance of 0.70 (±0.02) at 734nm and equilibrated at 30°C. For assay, 1.0mL of diluted ABTS solution was added to 1mL of each fungal dilution. The absorbance at 734nm was taken at 30°C exactly 30min. after mixing against solvent blank. The percentage inhibition of was calculated using the formula:

$$\text{Scavenging activity (\%)} = \left[1-\left(\frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) X\ 100\%\right]$$

(Butylated hydroxytoluene (BHT) was used as a standard antioxidant agent).

DPPH free radical scavenging activity

The free radical-scavenging activities of the previously prepared fungal dilutions were detected against DPPH (2,2-diphenyl-1-picrylhydrazyl) according to Mensor et al. (2001). One mL of each dilution was added to 1mL methanolic solution of 0.3mM DPPH. The mixture was shaken and left in a dark box for 30min. at room temperature (30±1°C). The blank of each sample was prepared with 2.0mL of sample solution with 1mL of methanol instead of DPPH, while 1mL of methanolic DPPH plus 2mL of methanol served as control. The absorbance of the resulting solution was measured at 517nm. The scavenging activity percentage of DPPH was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = \frac{\text{Control absorbance} - \text{sample absorbance)}}{\text{absorbance of control}} X\ 100\%$$

(Butylated hydroxytoluene (BHT) was used as a standard antioxidant agent).

Antibacterial test

The antibacterial activity of the fungal filtrates against some human pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus mutans*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*) was performed using the agar well diffusion method mentioned by Magaldi et al. (2004). Pure cultures of tested bacteria were sub-cultured using nutrient broth and incubated at 37°C on a rotary shaker at 120 rpm for 24h. Then, the intensity of different bacterial cells in the solutions were adjusted separately to obtain optical density 0.5 at 570nm using 0.9% saline solution. Each strain was swabbed uniformly on the individual Mueller-Hinton agar plates using sterile cotton swabs and equal size wells (5mm) were made in agar plates using gel puncture. Then the 100µL of different fungal filtrates were added into the respective wells. The zone of inhibition was measured using a zone scale after 24h incubation at 37°C.

Antitumor test

This test aimed to investigate the toxic activity of the tested fungal culture filtrates on two cancer human cell lines (CACO: colon cancer and MCF7: breast cancer). For cultural filtrate preparation, each fungal isolate was grown on potato dextrose broth medium for two weeks in dark at 28°C. The mycelial mat was removed by filtration through Whatman No.1 filter paper. Then, a toxicity test of the crude culture filtrate was done using the method of Kohler et al. (2005). The used cell lines were maintained in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics (penicillin/ streptomycin), to prevent bacterial contamination, and incubated at 37°C in 5% CO_2 before use. The medium was then replaced with fresh RPMI-10% FBS. The cells were maintained by subculturing them after reaching an acceptable confluence. The cells were put into 96-well cell culture plates at a concentration of 1×10^4 cells/mL and incubated for 24h under suitable conditions to reach logarithmic growth. The cells were exposed to different concentrations of tested fungal filtrates separately (0, 20, 40, 60, 80, and 100µg/mL), where 0µg/mL was considered the control. After the incubation periods (24 and 48h), the medium was discarded, and 5mg/mL of MTT reagent (yellow color) was added to each plate and incubated for 3–4h. This reagent detects the viable cells where it reduces inside its mitochondria to violet-colored formazan crystals. The formed formazan crystals were dissolved in 100µg acidified isopropanol and read at 630nm using an ELISA microplate reader (Bio-RAD microplate reader, Japan). Each treatment was repeated three times. The cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = (\text{Abs test}/ \text{Abs control}) \times 100,$$

where Abs is absorbance. Calculations of viable cell percentage gave an indication of cytotoxicity due to tested fungal filtrates. The obtained results were statistically analyzed using the parameters; mean, standard deviation (SD), and analysis of variance (ANOVA) using the online free program, Free Statistics Calculators version 4.0. Statistical significance was set at $P < 0.001$.

Results

Morphological identification of isolated fungi:

In this study, seven endophytic fungi were successfully isolated from roots of tomato plants, sub-cultured to pure cultures and named by the following abbreviations: Egy1-EU1, Egy2-EU2, Egy3-Neo, Egy4-YG, Egy5-SA1, Egy6-SA5 and Egy7-Gray (Photo 1). Description of morphological features of the cultures and the microscopic examination of diagnostic structures of each fungus was mentioned and discussed as follow where the obtained investigations showed that, six fungal isolates belong to Ascomycota while only one is Dematiaceous. The identification of the isolated fungi was recorded in Table 3.

Details of morphological and microscopic identification:

Aspergillus chevalieri:

Colonies growing fast on PDA plates attaining 5.5 cm in diameter after 5 days at 28°C. Surface floccose, margin thin and yellow color to brownish yellow at margin (Photo 2A&B). Reverse yellowish to brown under old regions at the center (Photo 2C). Globose to sub-globose cleistothecia abundant filled with numerous, hyaline, and globose to sub-globose asci (Photo 2D-F). Lenticular ascospores are recognized by smooth convex walls with recurved equatorial crests (Photo 2G). Anamorph: *Aspergillus chevalieri* is frequently appeared and characterized by long, smooth conidiophore terminated with sub-globose vesicle, conidial head uniseriate radiate (Photo 2H).

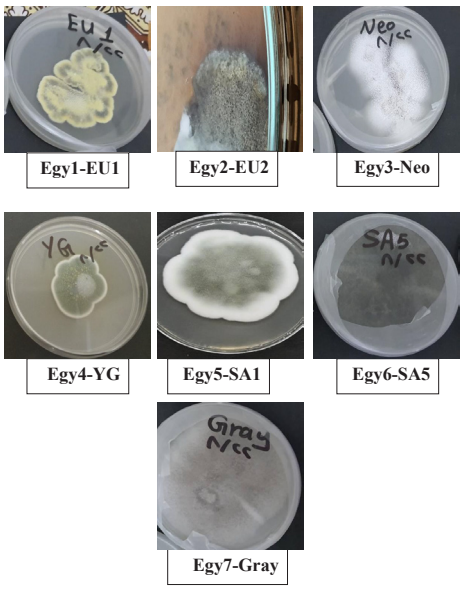


Photo 1. Pure cultures of the isolated endophytic fungi from roots of tomato plants

TABLE 3. Taxonomy and identification of the tested endophytic fungi

Taxonomical group	Code	Scientific name
Ascomycota	Egy1-EU1 and Egy2-EU2	*Aspergillus chevalieri*
	Egy3-NEO	*Talaromyces gossypi*i
	Egy4-YG	*Penicillium vanluykii*
	Egy5-SA1	*Penicillium capsulatu*m
	Egy7-Gray	(*Triangularia mangenoti*i)*
Dematiaceae	Egy6-SA5	(*Alternaria arborescens*)**

*: Not recognized by morphological and microscopic examination and identified by molecular identification.

**: Identified morphologically to only the genus level.

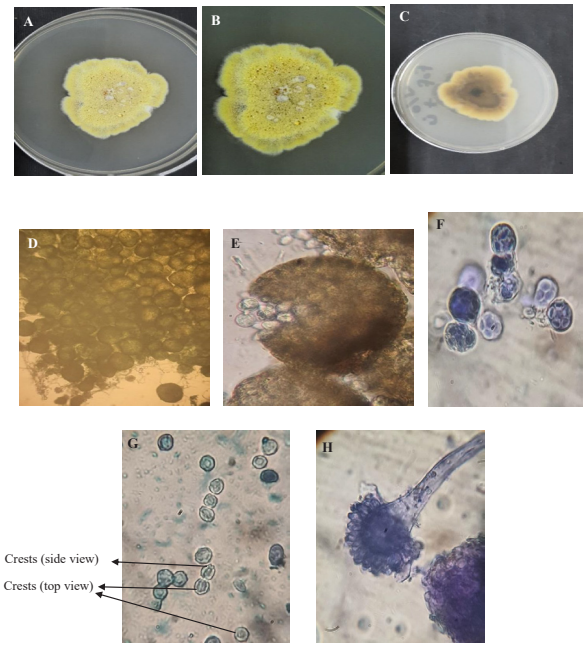


Photo 2. Macroscopic and microscopic features of *A. chevalieri*; appearance of culture on PDA (A&B), reverse (C), cleistocethia (D), enlarged broken cleistocethium showing asci (E), globose asci with ascospores appeared inside them (F), ascospores with distinct clear flexible crests (G) and anamorph stage (H)

Talaromyces gossypii

Characterized by white, floccose and slow-growing colonies on PDA plates reached 2 cm in diameter after 5 days at 28°C. Surface covered with colorless droplets exudate, reverse colorless tends to light pinkish at the center (Photo 3A&B). Cleistothecia are sub-globose and each one surrounded by loosely interwoven hyphae peridium (Photo 3C). Asci seems to be generated in chain, globose, sub-globose to elliptical (Photo 3D). Ascospores hyaline, smooth walled and elliptical **(**Photo 3E). *Penicillium* stage does not appeared during the microscopic examination.

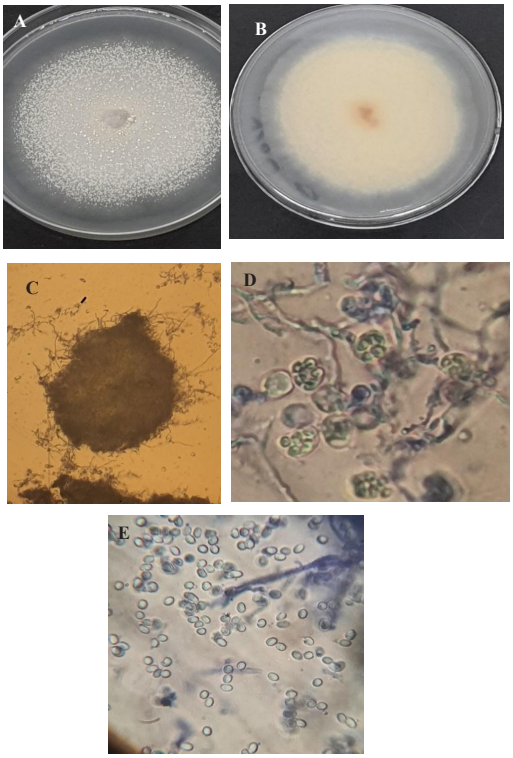


Photo 3. Macroscopic and microscopic features of *T. gossypii;* appearance of culture on PDA (A), reverse (B), cleistocethium (C), asci with ascospores appeared inside them (D) and ascospores (E)

Penicillium vanluykii

Colonies entire, velvety reached about 4.5 cm on PDA at 28°C after 5 days. Mycelium white appeared clearly at young area on margin while dense mass of dark green spores covered the remaining colony area, exudate droplets light yellow, reverse yellow with lighter yellow color beneath the margin (Photo 4A&B). Conidiophores smooth walled, branches divaricate and quarterverticillate, metulae present, phialides ampulliform, conidia globose to subglobose, smooth walled, distinct connectives appeared (Photo 4C-E).

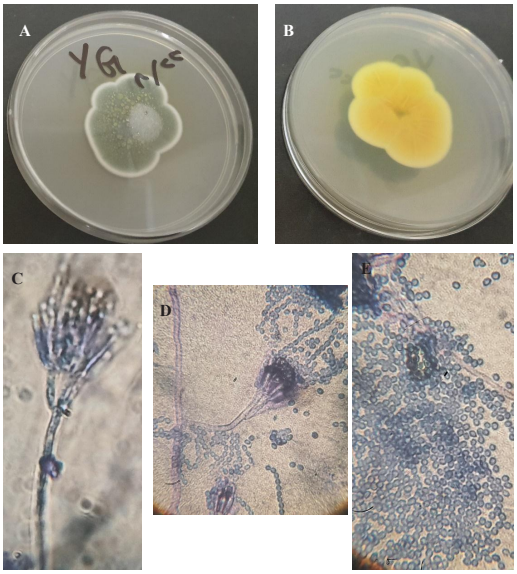


Photo 4. Macroscopic and microscopic features of *P. vanluykii;* appearance of culture on PDA (A), reverse (B), conidiophore (C&D) and conidia (E)

Penicillium capsulatum

Colony is very characteristic by its velutinous and cottony raised appearance, reached about 7cm after 5 days on PDA at 28°C. Color is white at the margins and greenish gray in the center, reverse creamish yellow beneath the old part of the colony and creamy under the margin. Conidiophores arising from aerial hyphae, short, smooth, and often monoverticillate, but irregularly metulate penecilli also found. Phialides in verticils of 5-8, ampulliform and acerose shaped. Conidia sub-globose to ellipsoidal with smooth walls (Photo 5A-C).

Triangularia mangenotii

This fungus was identified by molecular identification where the microscopic examination was not efficient to recognize it. The culture was characterized by its brownish gray color with black color reverse gradient to light brown to creamy beneath the margin of the colony (Photo 6 A&B). Microscopic examination showed true multicellular hyphae with numerous spherical to oval intercalary cells (Photo 6 C&D).

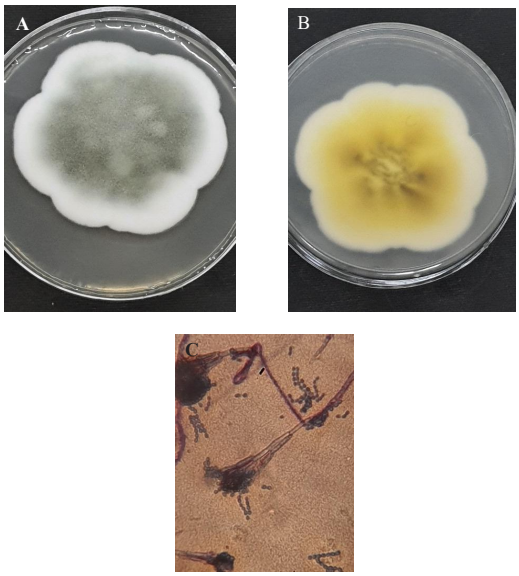
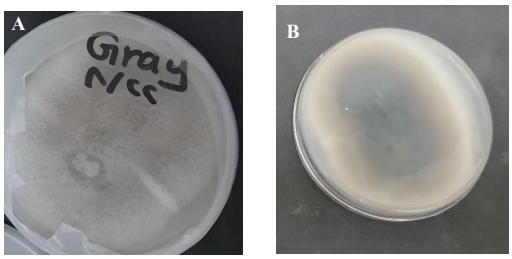


Photo 5. Macroscopic and microscopic features of *P. capsulatum;* appearance of culture on PDA (A), reverse (B), conidiophore and conidia (C)



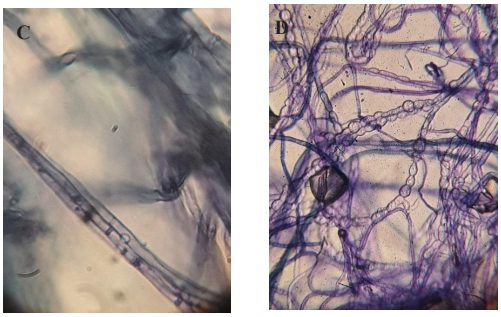


Photo 6. Macroscopic and microscopic features of *T. mangenotii;* appearance of culture on PDA (A), reverse (B), True hyphae (C) and rounded to oval intercalary cells (D)

Alternaria arborescens:

Morphology of the colony and microscopic examination retained this fungus to be identified as genus *Alternaria*. Colonies were characterized by effuse shaped, dark brownish black or black color with obvious brownish black reverse (Photo 7 A&B). Special shaped conidia of *Alternaria* were found by examination formed in chains, pyriform shaped with short peak, color is hyaline at first turned brown with age and smooth walled (Photo 7 C&D). Species is recognized by molecular identification as *A. arborescens* which belongs to section *A. alternata*.

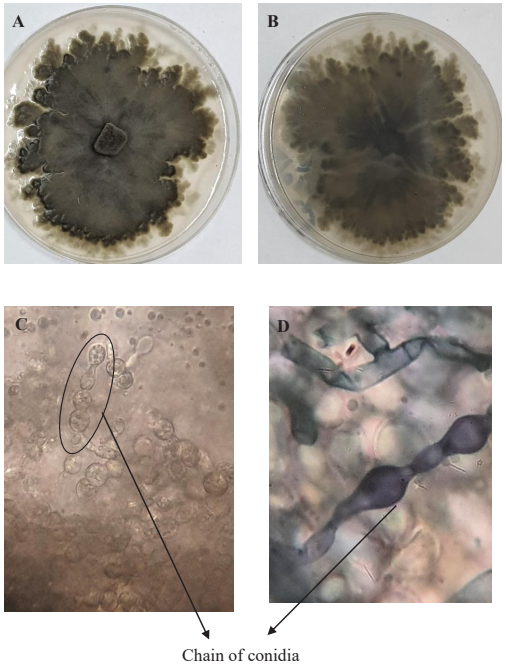


Photo 7. Macroscopic and microscopic features of *A. arborescens*; appearance of culture on PDA (A), reverse (B), chain of conidia (C&D)

Molecular characterization of tested fungi

Because certain fungal isolates were difficult to identified morphologically, DNA sequencing was recommended for fungus characterization. It was carried out for species conformation. Multiple species sequences were compared to analyze base pairs in the DNA sequence, and a phylogenic tree was constructed to study the phylogenic relationship of the distinct species. To molecular identification of the selected fungi, ITS and β-tubulin genes were successfully amplified and sequenced. The PCR amplification of ITS region of the seven isolates (Egy1-EU1, Egy2-EU2, Egy3-NEO, Egy4-YG, Egy5-SA1, Egy6-SA5 and Egy7-Gray) yielded PCR products ranged from 250 to 750bp, as shown in Photo 8A. The amplification products were sequenced for species identification. Also, PCR of β-tubulin gene indicated that seven isolates were positive with amplicon sizes of 480-600bp (Photo 8B).

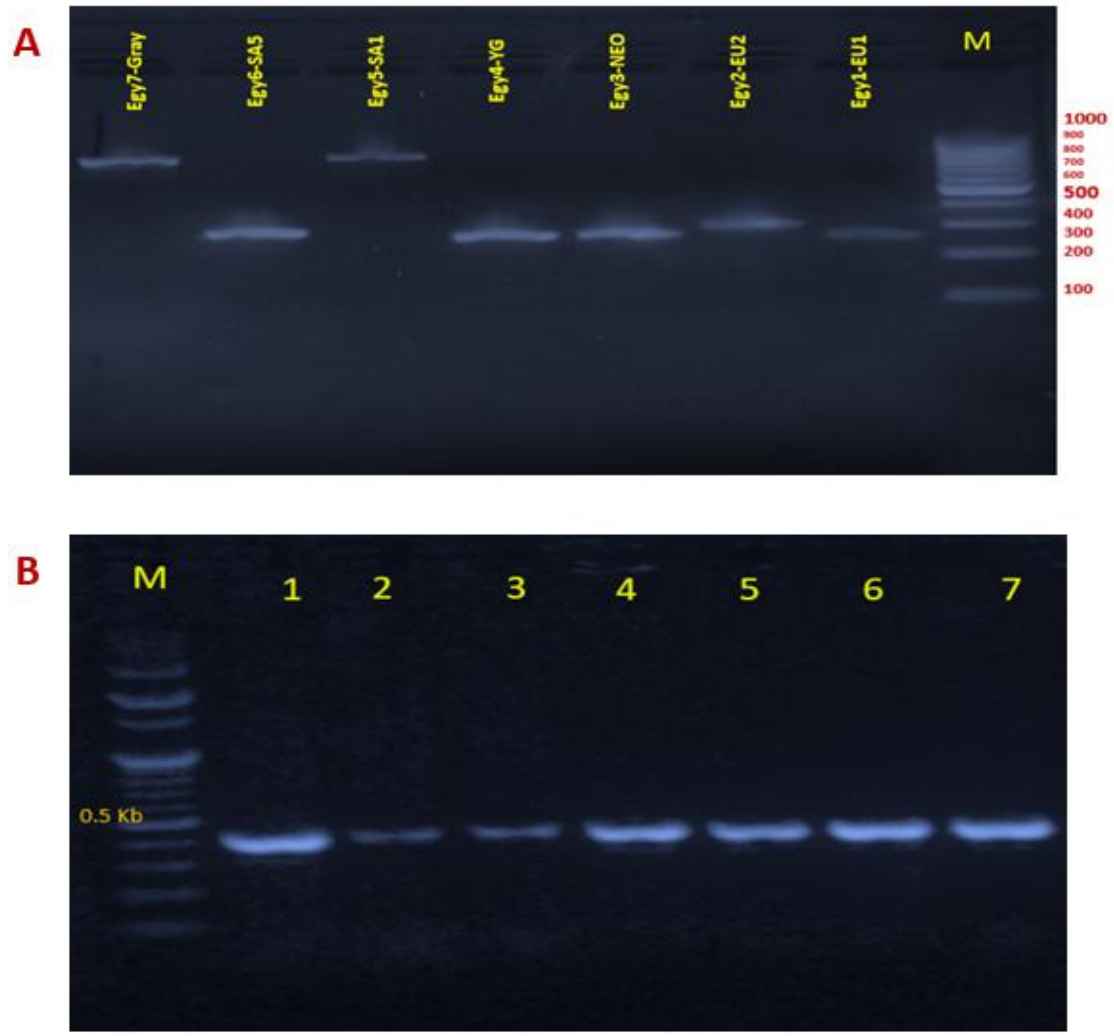


Photo 8. (A) PCR product profiles of ITS gene for selected isolates Lane (1): DNA marker 100bp, lane (2,8): isolates include Egy1-EU1, Egy2-EU2, Egy3-NEO, Egy4-YG, Egy5-SA1 , Egy6-SA5 and Egy7-Gray. (B) PCR product profiles of β-tubulin gene for selected isolates Lane (1): DNA marker 1kb, lane (1,7): include Egy1-EU1, Egy2-EU2, Egy3-NEO, Egy4-YG, Egy5-SA1 , Egy6-SA5 and Egy7-Gray isolates

The sequencing result of ITS and β-tubulin were edited and aligned using BLAST tool through the (NCBI) database to identify the possible genera of the isolates based on similarity as presented in Tables 4 and 5. Similarity search using BLAST analysis revealed that isolates (Egy1-EU1 and Egy2-EU2) belonged to the genus *Aspergillus* while, isolates (Egy4-YG and Egy5-SA1) belonged to the genus *Penicillium,* moreover, Egy3-NEO belong to the genus *Talaromyces*. Also, Egy6-SA5 belonged to the genus *Alternaria* and Egy7-Gray belonged to the genus *Triangularia.*

Multiple sequence alignment of ITS region results indicated that (Egy1-EU1 and Egy2-EU2) show 99.48% similarity with *Aspergillus chevalieri* (NR_135340.1), Egy3-NEO shows 99.52% similarity with *Talaromyces gossypii* (NR_147423.1), Egy4-YG shows 99.99% similarity with *Penicillium vanluykii* (NR_111813.1), Egy5-SA1 shows 99.67% similarity with *Penicillium capsulatum* (NR_121226.1), Egy6-SA5 shows 99.62% similarity with *Alternaria arborescens* (NR_135927.1) and Egy7-Gray shows 99.13% similarity with *Triangularia mangenotii* (NR_160111.1). Also, genetic distances between ITS aligned sequences of species were calculated and estimated by MEGA11 (Table 6).

TABLE 4. Multiple Sequence Alignment (MSA) of ITS gene and the accession numbers provided from NCBI for the submitted sequences

No	Isolate code	Significant alignments	E value	Per. Ident	Retrieved accession	Fungal strains	Submitted accession no.
1	Egy1-EU1	*Aspergillus chevalieri*	0.0	99.48	NR_135340.1	*Aspergillus chevalieri* Egy1-EU1	OP599904.1
		Aspergillus intermedius	0.0	99.30	NR_137448.1		
		Aspergillus costiformis	0.0	99.29	NR_135434.1		
		Aspergillus pseudoglaucus	0.0	98.43	NR_135336.1		
		Aspergillus proliferans	0.0	98.26	NR_135339.1		
		Aspergillus ruber	0.0	98.26	NR_131286.1		
2	Egy2-EU2	*Aspergillus chevalieri*	0.0	99.48	NR_135340.1	*Aspergillus chevalieri* Egy2-EU2	OP599905.1
		Aspergillus intermedius	0.0	99.30	NR_137448.1		
		Aspergillus costiformis	0.0	99.29	NR_135434.1		
		Aspergillus pseudoglaucus	0.0	98.43	NR_135336.1		
		Aspergillus proliferans	0.0	98.26	NR_135339.1		
3	Egy3-NEO	*Talaromyces gossypii*	0.0	99.52	NR_147423.1	*Talaromyces gossypii* strain Egy3-NEO	OP599906.1
		Talaromyces assiutensis	0.0	97.46	NR_172040.1		
		Talaromyces clemensii	0.0	91.19	NR_168822.1		
		Talaromyces udagawae	0.0	90.88	NR_145156.1		
		Talaromyces purpureus	0.0	90.42	NR_145153.1		
4	Egy4-YG	*Penicillium vanluykii*	0.0	99.99	NR_111813.1	*Penicillium vanluykii* strain Egy4-YG	OP599907.1
		Penicillium chrysogenum	0.0	99.83	NR_077145.1		
		Penicillium flavigenum	0.0	99.66	NR_103695.1		
		Penicillium rubens	0.0	99.66	NR_111815.1		
		Penicillium nalgiovense	0.0	99.49	NR_103694.1		
5	Egy5-SA1	*Penicillium capsulatum*	0.0	99.67	NR_121226.1	*Penicillium capsulatum* strain Egy5-SA1	OP599908.1
		Penicillium ramusculum	0.0	97.20	NR_121326.1		
		Penicillium hispanicum	0.0	93.39	NR_138307.1		
		Penicillium ornatum	0.0	92.69	NR_138306.1		
		Penicillium implicatum	0.0	93.36	NR_077160.1		
6	Egy6-SA5	*Alternaria arborescens*	0.0	99.62	NR_135927.1	*Alternaria arborescens* strain Egy5-SA5	OP599909.1
		Alternaria destruens	0.0	99.44	NR_137143.1		
		Alternaria eichhorniae	0.0	99.25	NR_111832.1		
		Alternaria cerealis	0.0	99.61	NR_136117.1		
		Alternaria betae-kenyensis	0.0	99.42	NR_136118.1		
7	Egy7-Gray	*Triangularia mangenotii*	0.0	99.13	NR_160111.1	*Triangularia mangenotii* Egy7-Gray	OP599910.1
		Zopfiella pilifera	0.0	98.91	NR_175137.1		
		Zopfiella marina	0.0	97.84	NR_103599.1		
		Cercophora dulciaquae	0.0	97.83	NR_177579.1		
		R. chlamydospora	0.0	88.57	NR_171803.1		

TABLE 5. Multiple Sequence Alignment (MSA) for β-tubulin and the accession numbers provided from NCBI for the submitted sequences

No	Isolate code	Significant alignments	E value	Per. Ident	Retrieved accession	Fungal strains	Submitted accession no.
1	Egy1-EU1	*Aspergillus chevalieri*	0.0	100.00	LC733668.1	*Aspergillus chevalieri* Egy1-EU1	LC733668.1
		Aspergillus chevalieri	0.0	100.00	KX455755.1		
		Aspergillus chevalieri	0.0	100.00	KU872178.1		
		Aspergillus chevalieri	0.0	99.57	AP024418.1		
		Aspergillus chevalieri	0.0	99.22	KU872171.1		
		Aspergillus chevalieri	0.0	99.10	MT410153.1		
2	Egy2-EU2	*Aspergillus chevalieri*	0.0	100.00	LC733669.1	*Aspergillus chevalieri* Egy2-EU2	LC733669.1
		Aspergillus chevalieri	0.0	100.00	KU872171.1		
		Aspergillus chevalieri	0.0	100.00	MT410153.1		
		Aspergillus chevalieri	0.0	100.00	MZ027912.1		
		Aspergillus chevalieri	0.0	100.00	KU872182.1		
3	Egy3-NEO	*Talaromyces gossypii*	0.0	100.00	LC733670.1	*Talaromyces gossypii* strain Egy3-NEO	LC733670.1
		Talaromyces assiutensis	0.0	100.00	KJ865720.1		
		Talaromyces assiutensis	0.0	100.00	KF114801.1		
		Talaromyces assiutensis	0.0	99.70	KM066124.1		
		Talaromyces assiutensis	0.0	99.70	MW727230.1		
4	Egy4-YG	*Penicillium vanluykii*	0.0	100.00	MK450923.1	*Penicillium vanluykii* strain Egy4-YG	LC733671.1
		Penicillium vanluykii	0.0	100.00	LC733671.1		
		Penicillium vanluykii	0.0	100.00	MK451173.1		
		Penicillium vanluykii	0.0	99.77	MK451167.1		
		Penicillium rubens	0.0	99.71	MF575007.1		
5	Egy5-SA1	*Penicillium capsulatum*	0.0	100.0	LC733672.1	*Penicillium capsulatum* strain Egy5-SA1	LC733672.1
		Penicillium capsulatum	0.0	97.09	MK951877.1		
		Penicillium sp.	0.0	93.87	JX140950.1		
		Penicillium canescens	0.0	93.86	EU427269.1		
		Penicillium ramusculum	0.0	94.76	ON561783.1		
6	Egy6-SA5	*Alternaria arborescens*	0.0	100.00	LC733673.1	*Alternaria arborescens* strain Egy5-SA5	LC733673.1
		Alternaria arborescens	0.0	99.87	MF070272.1		
		Alternaria arborescens	0.0	99.87	MF070265.1		
		Alternaria tenuissima	0.0	99.74	MF070250.1		
		Alternaria gaisen	0.0	99.74	MF070241.1		
7	Egy7-Gray	*Triangularia mangenotii*	0.0	100.00	LC733674.1	*Triangularia mangenotii* Egy7-Gray	LC733674.1
		Triangularia mangenotii	0.0	100.00	KP981571.1		
		Triangularia mangenotii	0.0	100.00	AY780142.1		
		Cercophora aff.	0.0	95.11	AY780105.1		
		Naviculispora terrestris	0.0	93.54	KP981567.1		

TABLE 6. Estimates of evolutionary divergence between the obtained ITS sequences of selected different seven fungal species

No	Organisms	Distances /similarity						
		1	2	3	4	5	6	7
1	OP599904.1_*Aspergillus_chevalieri*_strain_Egy1-EU1	**0.000**	98.90	82.30	88.71	86.70	68.70	69.10
2	OP599905.1_*Aspergillus_chevalieri*_strain_Egy1-EU2	0.011	**0.000**	82.10	88.71	86.44	69.23	68.22
3	OP599906.1_*Talaromyces_gossypii*_strain_Egy3-NEO	0.177	0.179	**0.000**	81.20	82.11	68.34	66.24
4	OP599907.1_*Penicillium_vanluykii*_strain_Egy4-YG	0.113	0.113	0.188	**0.000**	87.22	67.24	69.6
5	OP599908.1_*Penicillium_capsulatum*_strain_Egy5-SA1	0.133	0.136	0.172	0.119	**0.000**	65.55	69.4
6	OP599909.1_*Alternaria_arborescens*_strain_Egy5-SA5	0.306	0.313	0.324	0.328	0.357	**0.000**	66.2
7	OP599910.1_*Triangularia_mangenotii*_strain_Egy7-Gray	0.310	0.318	0.340	0.309	0.302	0.409	**0.000**

Phylogenetic analysis allowed us to associate seven endophytic fungi into five different clades. (Fig. 1). It was generated with a total of 46 sequences for ITS sequences. Clade I include two isolates (Egy1-EU1 and Egy2-EU2) were identified as *A. chevalieri*. Also, clade II represented with two isolates (Egy4-YG and Egy5-SA1) were identified as *P. vanluykii* and *P. capsulatum* respectively. In addition, isolate Egy6-SA5 was idendified as *A. arborescens*.

Moreover, multiple sequence alignment of β-tubulin (Table 5) indicated that both (Egy1 EU1and Egy2-EU2) show 100.0% similarity with *A. chevalieri* (KU872171.1), Egy3-NEO shows 99.52% similarity with *T. gossypii* (KJ865720.1), both (Egy4-YG and Egy5-SA1) shows 100.0% similarity with *P. vanluykii* (MK450923.1), and 99.09% similarity with *P.capsulatum* (MK951877.1). Egy6-SA5 shows 99.87% similarity with *A.arborescens* (MF070272.1) and Egy7-Gray shows 100% similarity with *T.mangenotii* (KP981571.1). Similarity, phylogenetic trees using β-tubulin sequences for the isolated species were constructed with 38 sequences. The phylogenetic tree (Fig. 2) showed that the lineages of the isolates could be grouped into five main clades, representing different genera (and species) as follows: *A. chevalieri* (Egy1-EU1 and Egy2-EU2), *Penicillium* (Egy4-YG and Egy5-SA1), *Talaromyces gossypii* (Egy3-NEO), *Alternaria* (Egy6-SA5), and *Triangularia* (Egy7-Gray). Also, amino acid variation and protein multiple sequence alignment between β-tubulin sequences of species were performed (Fig. 3).

The sequences were submitted to GenBank and received an accession numbers OP599904.1-OP599910.1 for ITS and (LC733668- LC733674) for β-tubulin gene. Our results showed that the ITS region and the adjacent rDNA could be ideal candidate DNA regions used for identifying these and related fungal species.

Genetic diversity of isolated fungal strains using ISSR marker

Molecular diversity of selected isolates was investigated by ISSR-PCR analysis approach, the results of ISSR analysis using six primers were illustrated in Photo 9. A DNA sample representing the seven fungal strains was subjected to PCR analysis using six ISSR primers. Data presented in Table 7 explained that polymorphism percentage (P%) and polymorphism information content (PIC) for pattern generated by ISSR marker. Results indicated that a total 180 fragments produced were obtained at different molecular weight ranged from 200 bp to 3000bp. All the 6 ISSR primers used for (seven fungal isolates) were found polymorphic, producing a total of 30 bands of which 10 bands were polymorphic whereas 20 bands were monomorphic. Polymorphism percentages (P%) of applied six primers were 60.00, 40.00, 40.00, 40.00, 0.00, and 16.66 % respectively. primer (1), have the highest (P%) with 60.00% Polymorphism. No unique bands produced by any primers. Also, PIC for ISSR primers (1, 2, 3, 4, 5 and 6) were calculated as 0.1625, 0.19375, 0.1500, 0.2000, 0.000 and 0.0625 generated from ISSR pattern. Principal component analysis (PCoA) is a powerful tool and the most basic multivariate data reduction

statistical technique. As shown in Fig. 4 B, Principal Correlation Analysis (PCoA) of isolated fungal strains using ISSR pattern showed two clouds A, and B. The numerical analysis clearly revealed two distinct clusters as shown in the dendrogram (Fig. 4A); it was classified into two main clusters (A and B). Cluster includes five isolates (EU1, EU2, NEO, YG and SA1), and cluster B include two isolates (SA5 and Gray).

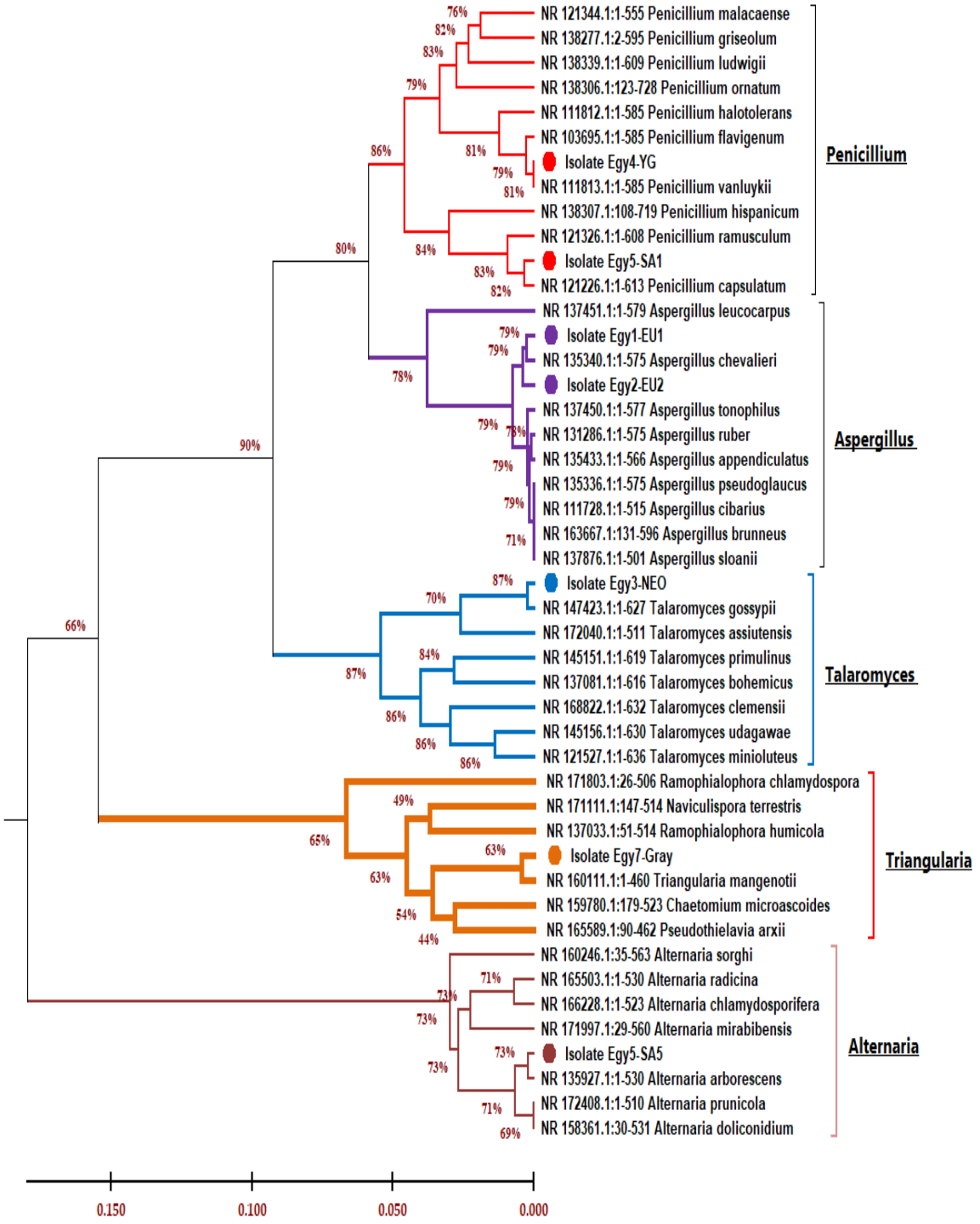


Fig. 1. Phylogenetic tree based on ITS sequences, showing the relationship between isolates and other species belong to its genus [The tree was constructed using the MEGA11 and neighbor-joining method]

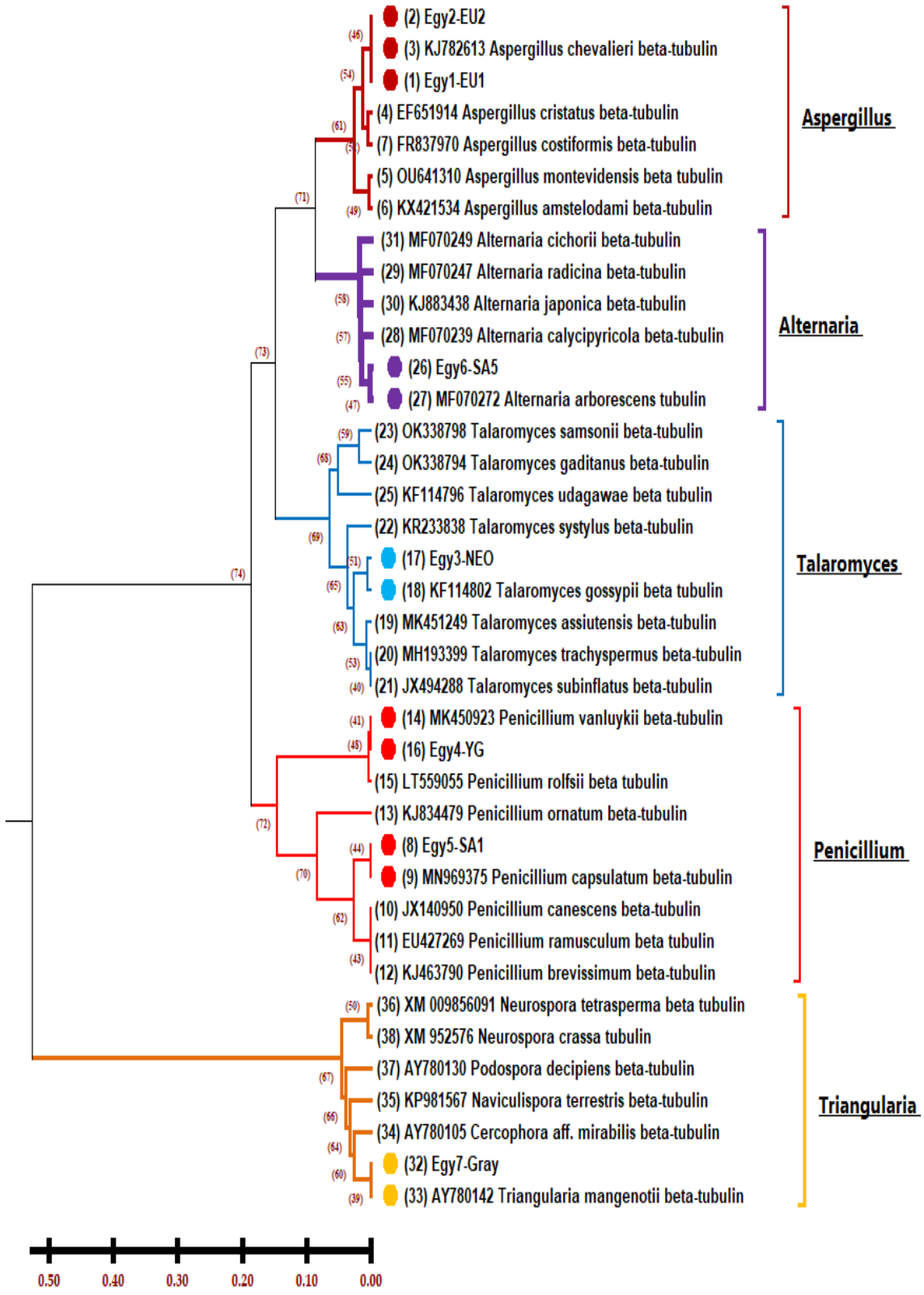


Fig. 2. Phylogenetic tree based on β-tubulin sequences, showing the relationship between isolates and other species belong to its genus [The tree was constructed using the MEGA11 and neighbor-joining method]

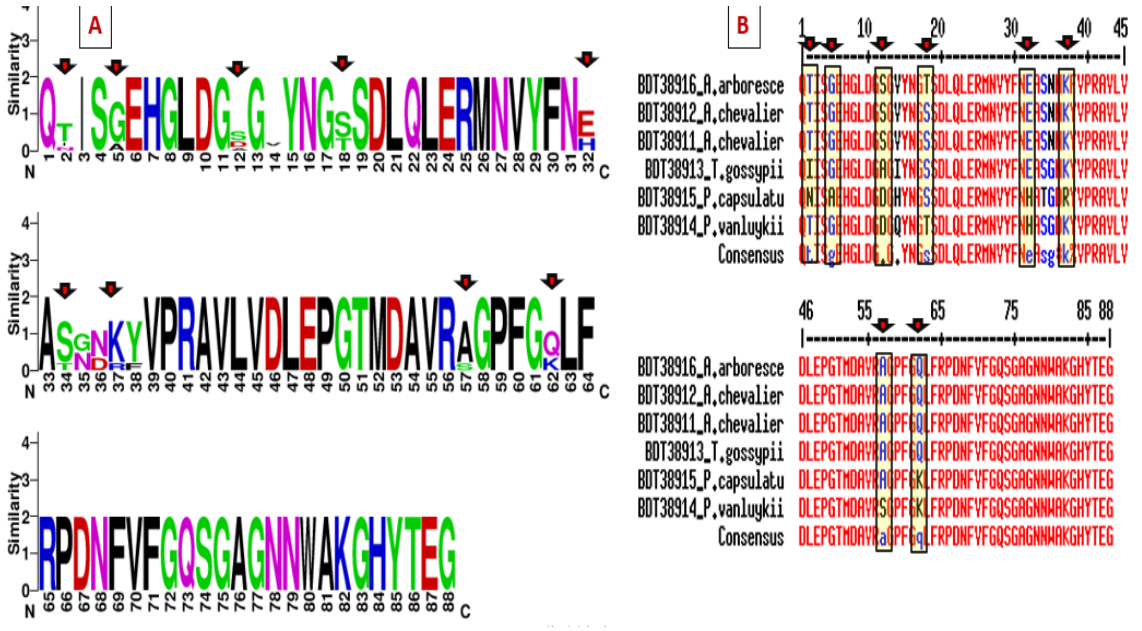


Fig. 3. (A) Amino acids variations of detected amino acid of the β-tubulin gene generated by WebLogo3 server. (B) Multiple amino acids alignments for selected fungal isolates generated by MultAlin server

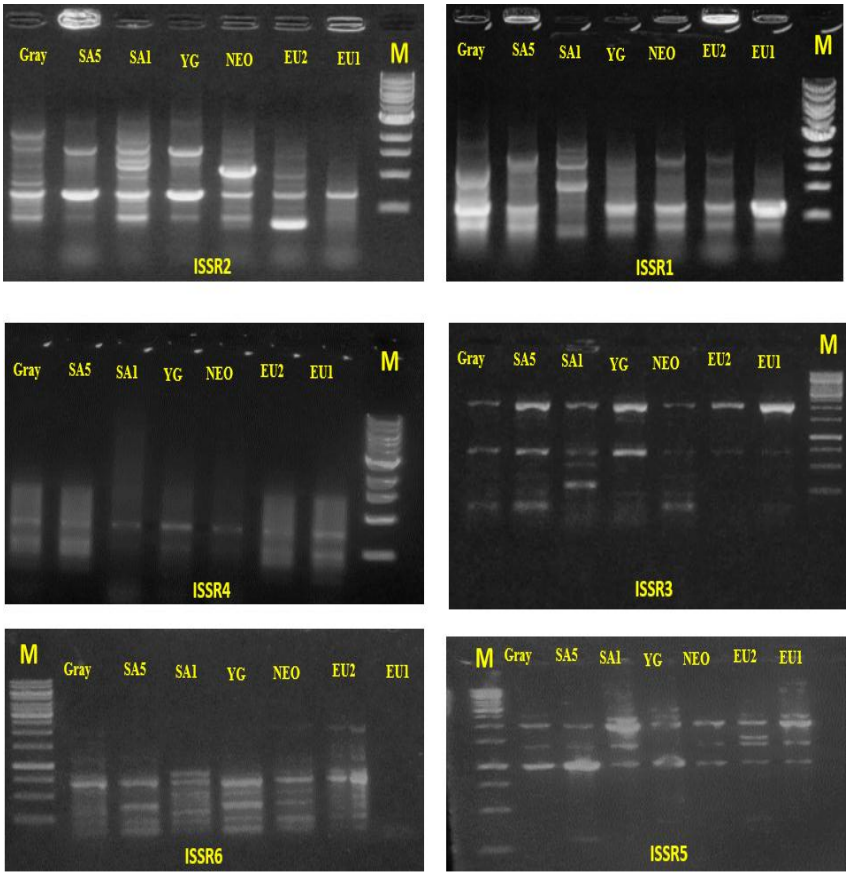


Photo 9. Patterns of ISSR electrophoresis of selected strains, Lane1: DNA ladder (PageRuler™ Plus Pertained), Lane (2-8): represent isolates from (2-8) represent as Egy1-EU1, Egy2-EU2, Egy3-NEO, Egy4-YG, Egy5-SA1, Egy6-SA5, and Egy7-Gray

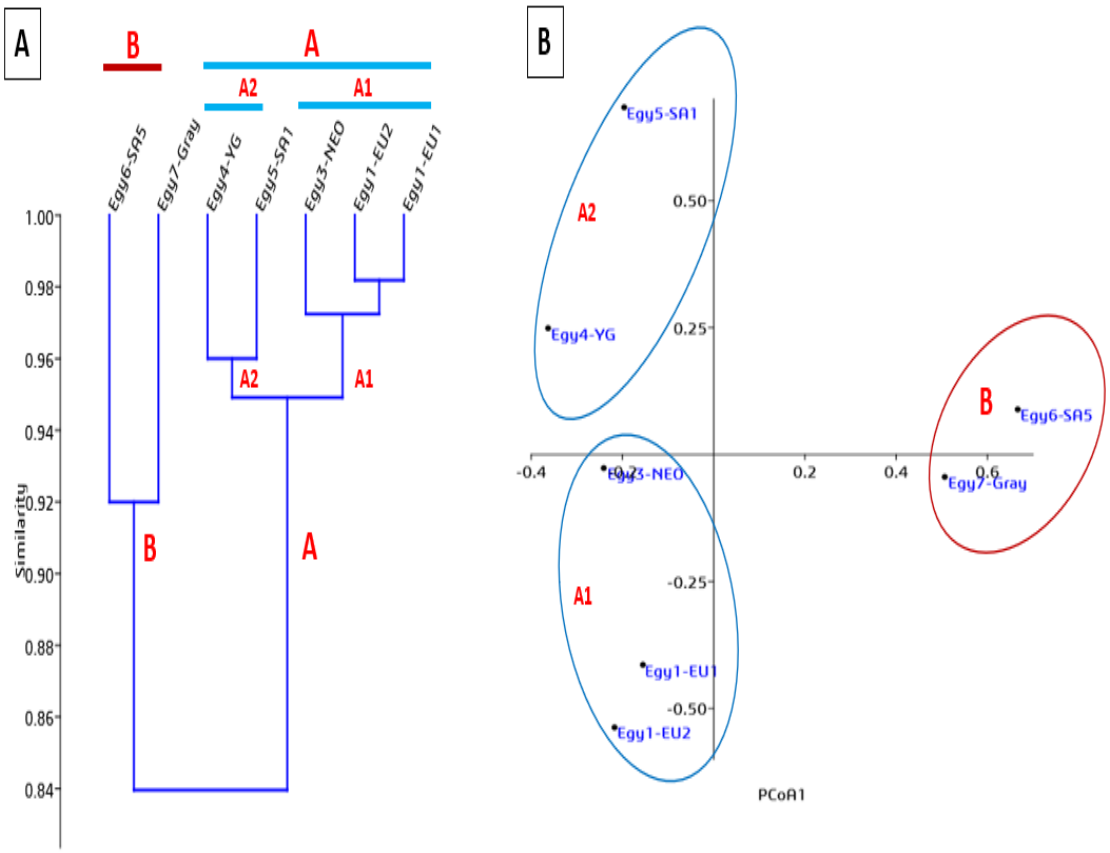


Fig. 4. (A) Dendrogram of ISSR-PCR pattern based on unweighted pair group method with arithmetic averages algorithm (UPGMA) for isolated fungal strain. (B) Principal component analysis (PCA) of isolated fungal strain

Antioxidant and antibacterial activities:

The antioxidant capacity of the tested fungi using different assays (DPPH, and ABTS) was evaluated where the BHT was used as a standard antioxidant agent. Results illustrated in Table 8 showed that all investigated assays were similarly dose dependent, increasing gradually with increasing concentration of fungal filtrate. DPPH radical scavenging activity (%) ranged from 18.09±2.4 to 91.96±1.1% at concentrations 0.25 and 1.0mL, respectively. Moreover, the ABTS radical scavenging activity followed the same trend and increased gradually from 5.88±0.4 to 83.11 ± 0.45 % by increasing filtrate concentrations from 0.25 to 1.0mL.

The test of antibacterial activity of the tested fungi revealed negative results against the used bacterial species (Table 9).

Antitumor test:

Different concentrations (20, 40, 60, 80 and 100 %) of culture filtrates of tested fungi separately were tested against CACO and MCF7 cancer cell lines where the IC_{50} was detected after 24 and 48h. This test was performed only by *A. chevalieri*, *T. gossypii*, *P. capsulatum* and *P. vanluykii* where the other two species (*T. mangenotii* and *A. arborescens*) were contaminated and not allowed to be detected. Tables 10 and 11 shown highly significant variations among the tested fungal filtrates against both cancer cell lines where the best observed results were reported after 48h. Highly antitumor activity was obtained by the culture filtrate of *P. vanluykii* against CACO cell line after 48 h where IC_{50} equals 6.3%. While, both filtrates of *A. chevalieri* and *P. capsulatum* recorded the lowest activity where, IC_{50} were 43.4 and 42% respectively followed by *T. gossypii*; 37% after 48h (Table 10). Resemble results were recorded for the tested fungi against MCF7 cell line where filtrate of *P. vanluykii* also exhibited the highest antitumor activity with IC_{50} equals 3.5% after 48h (Table 11).

TABLE 7. Polymorphism generated by ISSR pattern of the isolated fungal strains

Primer	MW	Egy1-EU1	Egy2-EU2	Egy3-NEO	Egy4-YG	Egy5-SA1	Egy6-SA5	Egy7-Gray	Frequency	Polymorphism	P. B	M.B	Unique	T. B	P%	PIC
ISSR1	**1200**	1	1	1	1	1	0	1	**87.5**	Polymorphic	3	2	0	5	60.00	0.1625
	750	1	1	1	1	1	1	1	**100**	Monomorphic						
	500	1	1	1	1	0	1	1	**87.5**	Polymorphic						
	400	1	1	1	1	1	1	1	**100**	Monomorphic						
	300	1	1	0	0	0	0	0	**25**	Polymorphic						
ISSR2	**2000**	1	1	1	1	0	**0**	**0**	**100**	Monomorphic	2	3	0	5	40.00	0.19375
	1500	1	1	1	1	0	**0**	**0**	**50**	Polymorphic						
	1000	1	0	1	0	1	1	**1**	**62.5**	Polymorphic						
	700	1	1	1	1	1	1	1	**100**	Monomorphic						
	400	1	1	1	1	1	1	1	**100**	Monomorphic						
ISSR3	**2500**	1	1	1	1	1	1	1	**100**	Monomorphic	2	3	0	5	40.00	0.1500
	750	1	1	1	1	1	1	1	**100**	Monomorphic						
	600	0	0	1	1	1	1	1	**75**	Polymorphic						
	500	0	0	1	1	1	1	1	**75**	Polymorphic						
	250	1	1	1	1	1	1	1	**100**	Monomorphic						
ISSR4	**1000**	1	1	0	0	0	1	1	**50**	Polymorphic	2	3	0	5	40.00	0.2000
	800	1	1	1	1	1	1	1	**100**	Monomorphic						
	600	1	1	1	1	1	1	1	**100**	Monomorphic						
	500	1	1	1	1	1	1	1	**100**	Monomorphic						
	250	1	1	0	0	0	1	1	**50**	Polymorphic						
ISSR5	**3000**	1	1	1	1	1	1	1	**100**	Monomorphic	0	4	0	4	0.00	0.000
	2500	1	1	1	1	1	1	1	**100**	Monomorphic						
	1500	1	1	1	1	1	1	1	**100**	Monomorphic						
	1000	1	1	1	1	1	1	1	**100**	Monomorphic						
ISSR6	**1500**	1	1	0	0	0	0	0	**25**	Polymorphic	1	5	0	6	16.66	0.0625
	1000	1	1	1	1	1	1	1	**100**	Monomorphic						
	750	1	1	1	1	1	1	1	100	Monomorphic						
	500	1	1	1	1	1	1	1	**100**	Monomorphic						
	250	1	1	1	1	1	1	1	100	Monomorphic						
	200	1	1	1	1	1	1	1	**100**	Monomorphic						
Total		**28**	**27**	**26**	**25**	**23**	**25**	**26**		**180**	**10**	**20**	**0**	**30**	**44**	

PB number of polymorphic bands, MB number of monomorphic bands, TB number of total bands, %P percent polymorphism (PIC) polymorphism information content.

TABLE 8. Antioxidant activity of fungal filtrates

No	Strain	Antioxidant activity					
		DPPH%			ABTS%		
		1mL	0.5mL	0.25mL	1mL	0.5mL	0.25mL
1	(BHT)	95.99±0.8	75.62±1.8	58.88±1.8	93.43 ± 0.33	66.89 ± 1.5	25.85± 0.01
2	*A. chevalieri* Egy1-EU1	89.98±0.7	65.76±5.7	25.64±0.9	80.57 ± 0.19	33.57 ± 0.19	5.88±0.4
3	*A. chevalieri* Egy2-EU2	91.83±1.3	59.63±3.2	21.06±2.0	83.11 ± 0.45	40.88 ± 0.45	11.36±0.1
4	*T. gossypii* Egy3-NEO	91.96±1.1	62.18±8.1	18.46±1.5	42.64 ± 0.30	16.45 ± 0.30	0.00
5	*P. vanluykii* Egy4-YG	84.66±3.4	61.90±4.3	23.91±3.2	29.77 ± 0.29	8.21 ± 0.29	0.00
6	*P. capsulatum* Egy5-SA1	89.72±1.1	52.91±1.8	18.09±2.4	63.18 ± 0.28	23.99 ± 0.44	0.00
7	*A.arborescens* Egy5-SA5	91.70±1.1	53.07±1.4	22.09±2.4	55.41± 0.28	19.67± 0.51	0.00
8	*T. mangenotii* Egy7-Gray	91.00±1.4	55.39±8.7	21.11±2.9	41.21 ± 0.28	12.34 ± 0.29	0.00

TABLE 9. Antibacterial activity of fungal filtrates

No	Strain	Antibacterial activity				
		E. coli G-ve	*S. aureus* G+ve	*S. mutans* G+ve	*E. faecalis* G+ve	*P. aeruginosa* G-ve
1	*A. chevalieri* Egy1-EU1	(-)	(-)	(-)	(-)	(-)
2	*A. chevalieri* Egy2-EU2	(-)	(-)	(-)	(-)	(-)
3	*T. gossypii* Egy3-NEO	(-)	(-)	(-)	(-)	(-)
4	*P. vanluykii* Egy4-YG	(-)	(-)	(-)	(-)	(-)
5	*P. capsulatum* Egy5-SA1	(-)	(-)	(-)	(-)	(-)
6	*A.arborescens* Egy5-SA5	(-)	(-)	(-)	(-)	(-)
7	*T. mangenotii* Egy7-Gray	(-)	(-)	(-)	(-)	(-)

TABLE 10. Cytotoxicity of colon cancer cell line (CACO) after 24 and 48 h at different concentrations of cultural filtrate of the tested endophytic fungi

Conc. (%)	Abs. after 24h (Mean ± SD)				Abs. after 48h (Mean ± SD)			
	Egy1-EU1	Egy3-NEO	Egy4-YG	Egy5-SA1	Egy1-EU1	Egy3-NEO	Egy4-YG	Egy5-SA1
Control	3.54± 0.08	3.54± 0.08	3.54± 0.08	3.54± 0.08	1.56± 0.067	1.56± 0.067	1.56± 0.067	1.56± 0.067
20	3.2± 0.067	3.257± 0.119	1.087± 0.42	3.287± 0.06	1.3± 0.027	1.32± 0.15	0.26± 0.076	1.26± 0.1
40	3.03± 0.07	3.03± 0.005	0.079± 0.001	3.14± 0.028	1.135± 0.069	0.88± 0.098	0.096± 0.0018	0.91± 0.07
60	0.61± 0.65	1.105± 0.145	0.06± 0.002	1.87± 0.148	0.198± 0.017	0.39± 0.056	0.084± 0.002	0.8± 0.02
80	0.135± 0.002	0.469± 0.347	0.057± 0.003	0.25± 0.09	0.14± 0.006	0.12± 0.004	0.08± 0.002	0.39± 0.077
100	0.121± 0.006	0.135± 0.004	0.052± 0.002	0.105± 0.005	0.123± 0.01	0.15± 0.02	0.076± 0.002	0.1± 0.007
IC_{50} (%)	48.2	51.5	17	58.5	43.4	37	6.3	42
F-Value ANOVA:	12.037				12.72			
P- value	0.00003				0.00002			

Egy1-EU1: *Aspergillus chevalieri*, Egy3-NEO*: Talaromyces gossypii*, Egy4-YG*: Penicillium vanluykii* and Egy5-SA1: *Penicillium capsulatum*. Abs.: Absorbance. SD: Standard Deviation.

TABLE 11. Cytotoxicity of breast cancer cell line (MCF7) after 24 and 48 h at different concentrations of cultural filtrate of the tested endophytic fungi

Conc. (%)	Abs. after 24h (Mean ± SD)				Abs. after 48h (Mean ± SD)			
	Egy1-EU1	Egy3-NEO	Egy4-YG	Egy5-SA1	Egy1-EU1	Egy3-NEO	Egy4-YG	Egy5-SA1
Control	0.48± 0.04	0.48± 0.04	0.48± 0.04	0.48± 0.04	0.63± 0.008	0.63± 0.008	0.63± 0.008	0.63± 0.008
20	0.35± 0.04	0.4± 0.007	0.17± 0.01	0.4± 0.026	0.26± 0.06	0.47± 0.16	0.17± 0.06	0.466± 0.057
40	0.18± 0.006	0.34± 0.023	0.12± 0.014	0.29± 0.012	0.13± 0.013	0.47± 0.05	0.08± 0.001	0.333± 0.04
60	0.12± 0.011	0.12± 0.0004	0.09± 0.0001	0.23± 0.017	0.1± 0.0006	0.19± 0.035	0.07± 0.001	0.15± 0.01
80	0.11± 0.004	0.106± 0.002	0.09± 0.001	0.1± 0.005	0.095± 0.003	0.124± 0.017	0.066± 0.003	0.088± 0.012
100	0.1± 0.006	0.1± 0.002	0.085± 0.005	0.087± 0.004	0.089± 0.0008	0.079± 0.004	0.054± 0.002	0.056± 0.004
IC_{50} (%)	22.6	34.4	5.6	34.5	14.88	21.99	3.5	32.28
F-Value ANOVA: P- value	21.43 0				18.23 0			

Egy1-EU1: *Aspergillus chevalieri*, Egy3-NEO*: Talaromyces gossypii*, Egy4-YG*: Penicillium vanluykii* and Egy5-SA1: *Penicillium capsulatum*. Abs.: Absorbance. SD: Standard Deviation.

Discussion

The present work introduced seven entophytic fungi which isolated from roots of tomato plants suffered from extreme hot temperature in the summer season and infected with the plant pathogenic fungus, *Fusarium*. The isolated fungi were identified morphologically where six of them are ascomycetes belong to genera, *Alternaria*, *Aspergillus*, *Penicillium* and *Talaromyces* while the seventh genus is *Triangularia*. The detailed microscopic features of each fungus were examined and compared with the documented characteristics in the identification books and literatures. The description of *A. chevalieri* mentioned here is agreed and supported by Hong et al. (2011). On the other hand, the presence of colorless droplets exudate and colorless reverse tends to light pinkish at the center of *T. gossypii* culture in the plates was disagreed the findings of Yilmaz et al. (2014), who reported that exudates absent and reverse is pale orange to brownish orange, but, the authors predicted that this difference might be due to type of used media. While the examined features of ascomata containing asci and ascospores were liked those described in Moubasher (1993). Moreover, the morphological appearance and microscopic description of the other isolates; *A. arborescens*, *P. capsulatum* and *P. vanluykii* was agreed with the characteristic features reported in the previous studies (Ali et al., 2006; Houbraken et al., 2012; Woudenberg et al., 2013).

Also, the study exhibited molecular identification of the isolated fungi using different molecular markers which considered rare approach in Egypt. The isolated fungal isolates were further subjected to PCR amplification coupled with DNA sequencing of two molecular genes markers. Sequence results of the fungal species of genera: *Aspergillus*, *Penicillium*, *Alternaria*, and *Talaromyces* revealed above 95% similarities with the recorded isolates in the GenBank. The identification and molecular characterization of the fungal isolates to specie level gave a better result by PCR amplification and sequencing of ITS region, and β-tubulin genes. Therefore, ITS region and β-tubulin were recommended be used as molecular markers for species level identification of fungi. many other researchers have also used the ITS as a fungal barcoding gene for fungal identification. For

example, a previous study of Nour et al. (2022) employed the ITS gene for the identification of some *A. terreus* isolates.

The results of PCoA revealed that two out of seven principal components were significant (Eigen value >1) and contributed 62.88% of the total variation. PCo1 accounted for 38.33% and PCo2 accounted for 24.55% of the total variation. PCoA found the same grouping pattern as found in the cluster analysis, indicating that significant variation exists in this study, the separation of PCo1 and PCo2 showed that seven fungal isolates were dispersed in all quarters, indicating a high level of genotypic variation among the fungal isolates. These results were similar to Flórez et al. (2007) who reported that genetic diversity found among the different *P. roqueforti* and *G. candidum* isolates was assessed by Rapid amplified polymeric DNA-polymerase chain reaction (RAPD-PCR) with two primers.

All the tested fungal species revealed positive antioxidant activities which in agreement with many other studies on extracts have found a positive and highly significant of antioxidant activity due to a high concentration of phenolic content (Lin et al., 2018). Also, our results were agreed with Mollaei et al. (2019) who obtained antioxidant activity on extracts of endophytic fungi of *Fusarium tricinctum* and *Alternaria altenata*. In addition the negative results of the tested fungal extracts against some bacterial strains are disagree with Farhat et al. (2022) who isolated endophytic fungi which showed strong antibacterial activity against pathogenic bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Bacillus subtilis* and *Escherichia coli*).

The present findings indicated that the culture filtrate of *P. vanluykii* exhibited a high antitumor activity against both CACO and MCF7 cell lines which gives a glimmer of hope for using this fungal filtrate for this purpose. Further studies still needed in this point. By research, there are no previous studies reported the cytotoxicity and antitumor activity of *P. vanluykii* and *P. capsulatum* which means that our study is considered the first one recorded that finding. While there were many other literatures investigated the cytotoxicity activity of *A. chevalieri* against various cancer cell lines (Kanokmedhakul et al., 2011; Vasarri et al., 2022). On the other hand, using the MTT assay as an accurate and a rapid tool for assaying the cytotoxicity when exposed to toxic materials have been recommended by many researchers (Visconti et al. 1991; Kitabatake et al., 1993; Hanelt et al., 1994; Widestrand et al., 2003).

Conclusions

The present work introduced seven entophytic fungi which isolated from roots of tomato plants suffered from extreme hot temperature and *Fusarium* infection. The isolated fungi were identified morphologically where six of them are ascomycetes belong to genera, *Alternaria*, *Aspergillus*, *Penicillium* and *Talaromyces* while the seventh genus is *Triangularia*. Also, the study exhibited molecular identification of fungi using different molecular markers which considered rare approach in Egypt. The isolated fungal isolates were further subjected to PCR amplification coupled with DNA sequencing of two molecular genes markers. Sequence results of the fungal species of genera: *Aspergillus*, *Penicillium*, *Alternaria*, and *Talaromyces* revealed above 95% similarities with the recorded isolates in the GenBank. The identification and molecular characterization of the fungal isolates to specie level gave a better result by PCR amplification and sequencing of ITS region, and β-tubulin genes. Therefore, ITS region and β-tubulin were recommended be used as molecular markers for species level identification of fungi.

Acknowledgment: Authors thank Botany and Microbiology Department, Faculty of Science, Tanta University, Egypt and Microbial Genetics, Biotechnology Research Institute, National Research Centre, Dokki, Giza, Egypt.

Competing interests: The authors report no conflicts of interest regarding this work.

Authors' contributions: All authors contributed to the study conception and design. Samah A. El-Debaiky designed experiments, performed isolation, morphological identification, microscopic examination and photographed and antitumor activities. Ahmed F. El-Sayed carried out the molecular barcoding, genetic diversity, phylogenetic analysis, antioxidant, and antibacterial activity.

Ethics approval: Not applicable.

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التعريف المورفولوجي والجزيئي للفطريات الداخلية من جذور الطماطم وتقييم أنشطتها المضادة للأكسدة والسمية للخلايا

سماح عبد القادر الدبيكى[1]، أحمد فكرى السيد[2، 3]

[1]قسم النبات والميكروبيولوجى- كلية العلوم- جامعة طنطا - طنطا – مصر، [2]قسم الوراثة الميكروبية- معهد بحوث التكنولوجيا الحيوية- المركز القومي للبحوث- الجيزة- مصر، [3]المركز المصري للبحوث والطب التجديدي- القاهرة- مصر.

تعرف الفطريات الداخلية على أنها مجموعه من الفطريات الأسكية التي تعيش بدون أعراض في الأنسجة الداخلية لبعض النباتات. وتعتبر لها دور مفيد في حماية النبات حيث تقوم بإنتاج العديد من المنتجات الطبيعية النشطة بيولوجيًا. لذلك هدفت الدراسة الحالية إلى عزل أنواع الفطريات الداخلية المرتبطة بجذور نباتات الطماطم والحصول عليها في صورة مزارع نقية والتعرف عليها حسب خصائصها المورفولوجية والوراثية. قدمت النتائج سبعة أنواع فطرية تنتمي إلى أجناس *Alternaria*، *Aspergillus*، *Penicillium*، *Talaromyces* و *Triangularia* والتي تم تعريفها وفقًا للوصف الظاهرى للمستعمرات الفطرية وكذلك الفحص المجهري الدقيق لتركيب كل فطر. تم تأكيد التعريف من خلال التوصيف الجزيئي باستخدام تسلسل جينات ITS و β-tubulin ثم تم إيداعها في GenBank بأرقام انضمام متسلسلة من OP599904.1 إلى OP599910.1 لـ ITS ومن LC733668 إلى LC733674 لـ β-tubulin. بالإضافة إلى ذلك، أظهرت الدراسات المضادة للأكسدة والبكتيريا ومضادات الأورام للفطريات الداخلية المعزولة إمكانات عالية لمضادات الأكسدة ونتائج سلبية ضد عزلات البكتريا المستخدمة وأنشطة ناجحة مضادة للأورام.