International Journal of Applied Sciences and Biotechnology

A Rapid Publishing Journal

ISSN 2091-2609

Available online at:

http://www.ijasbt.org

&

http://www.nepjol.info/index.php/IJASBT/index

Indexing and Abstracting

CrossRef, Google Scholar, Global Impact Factor, Genamics, Index Copernicus, Directory of Open Access Journals, WorldCat, Electronic Journals Library (EZB), Universitätsbibliothek Leipzig, Hamburg University, UTS (University of Technology, Sydney): Library, International Society of Universal Research in Sciences (EyeSource), Journal Seeker, WZB, Socolar, BioRes, Indian Science, Jadoun Science, Jour-Informatics, Journal Directory, JournalTOCs, Academic Journals Database, Journal Quality Evaluation Report, PDOAJ, Science Central, Journal Impact Factor, NewJour, Open Science Directory, Directory of Research Journals Indexing, Open Access Library, International Impact Factor Services, SciSeek, Cabell's Directories, Scientific Indexing Services, CiteFactor, UniSA Library, InfoBase Index, Infomine, Getinfo, Open Academic Journals Index, HINARI, etc.

CODEN (Chemical Abstract Services, USA): IJASKD

Vol-2(2) June, 2014

IC Value: **4.37**

For any type of query and/or feedback don't hesitate to email us at: editor.ijasbt@gmail.com

ISSN (Online): 2091-2609
DOI Prefix: 10.3126/ijasbt

International Journal of Applied Sciences and Biotechnology
(IJASBT)

Research Article

PHYLOGENY AND ANTAGONISTIC ACTIVITY OF SOME PROTOPLAST FUSANTS IN *TRICHODERMA* AND *HYPOCREA*

Mohamed M. Hassan*[1,2], Ismail A. Ismail[1,3] and Abd El-Latif A. Sorour[1]

[1]Scientific Research Center, Biotechnology and Genetic Engineering Unit, Taif University, KSA
[2]Genetics Department, Faculty of Agriculture, Minufiya University, Egypt
[3]Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Giza Egypt.

Corresponding author email: khyate_99@yahoo.com

Abstract

The present work aimed to apply mutagenesis and inter-specific protoplast fusion techniques of two locally isolated *Hypocrea* and *Trichoderma* to enhancement their biocontrol abilities against some important plant fungal pathogens which cause damping-off diseases that attacking different crops. The mutants were selected after EMS/UV treatment of *Trichoderma* isolates. The obtained protoplasts were fused by polyethylene glycol, and six fusants were selected for further studies. The phylogeny of the parental strains was carried out using sequence of ITS region. The BLAST of the obtained sequence was identified these isolates as *H. koningii* and *T. asperellum*. The fused protoplasts of the two mutant strains have been regenerated on PDA medium supplemented with the two fungicides. Most of the fusants exhibited fast mycelial growth on PDA as compared to parent strains. The obtained results indicated that partial or incomplete genetic recombination may be possible during nuclear and cytoplasmic protoplast fusion. Most of the fusants have shown powerful antagonistic activity against the grapevine pathogens as *Pythium ultimum* and *Fusarium roseum* as shown in dual culture and observed by SEM technique. Results of the present study demonstrated the scope and significance of the protoplast fusion technique, which can be used to develop superior hybrid strains of filamentous fungi that absent sexual stages in *Trichoderma* enhance biological control activity.

Key words: Protoplast fusion; ITS sequence; SEM technique; *Trichoderma*.

Introduction

More than 200 species of *Hypocrea* have been described, yet the genus has never been monographed. Recent systematic research suggests that it is not possible to identify a *Hypocrea* species unless its *Trichoderma* anamorph is known (Chaverri *et al*. 2003). Although only about 50 species of *Trichoderma* have been described, exploration of new geographical locations and ecological niches has revealed many additional under scribed species. *H. koningii* and *T. asperellum* are known producer of cellulolytic enzymes Feng *et al*., (2011), that are extensively used for the degradation and other processes of cellulose materials particularly in textile and paper industries besides, it is also used for wastewater treatment. Fungal protoplasts are important tools in physiological and genetic research, as well as genetic manipulation which can be successfully achieved through fusion of protoplasts in filamentous fungi that lack the capacity for sexual reproduction (Hayat and Christias 2010). Hence, protoplast fusion is one of the important approaches in the strain improvement programme Hassan, *et al*., (2011). However, limited attempts were made to improve the strain of *Trichoderma* to enhance enzyme production. However, limited attempts were made to improve the strain of *Trichoderma* and *Hypocrea* to enhance antagonistic activity especially against some grapevine pathogens. Several molecular techniques used to characterize fungi species were reported, including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and sequence analysis of the nuclear ribosomal internal transcribed spacer of rDNA (ITS-rDNA region) is one of the famous methods among these molecular characterization techniques (Sharma and Shanmugam, 2011; Hassan *et al*., 2013), with this background, the present work was aimed to (1) isolation and molecular identification of *Trichoderma* and *Hypocrea* isolates obtained from grapevine farmers in Taif Government. (2) Isolate and fuse the protoplasts from *H. koningii* and *T. asperellum* (3) Evaluate the fusants antagonistic activity against some grapevine pathogens such as *Pythium ultimum* and *Fusarium roseum* using dual culture and SEM techniques.

Materials and Methods

Trichoderma isolation and mutants induced

The two parental strains *H. koningii* and *T. asperellum* were used in this study isolated from grapevine farms in Taif, KSA according to diluted method. Fungicide tolerant mutants were induced by EMS/UV treatment, according to Salama and Tolba (2003).

Genomic DNA Isolation

Fungal mycelia of the selected fungus strains were inoculated onto PDA broth for five days. Genomic DNA for each *Trichoderma* strain was extracted using DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions.

Molecular characterization

ITS1 and ITS2 regions together with 5.8S gene in rRNA from *H. koningii* and *T. asperellum* were amplified as designed by Hermosa *et al*., (2000). The nucleotide sequences of 5.8S-ITS region were determined using the sequencer (Gene analyzer 3121). The sequencing data were compared against the Gene Bank database (http://www.ncbi.nlm.nih.gov/BLAST/), where a nucleotide blast program was chosen to identify the homology between the PCR fragments and the sequences on the Gene Bank database.

Protoplast preparation and fusion

Protoplasts preparation was carried out using fungicide tolerant isolates of *Trichoderma* according to Stasz *et al.,* (1988). Protoplasts were fused using a procedure similar to that described by Pe′er and Chet (1990). 1 ml of the suspension containing 10^6 protoplasts in STC buffer (0.6 M sorbitol; 10 mM CaCl2; 10 mM Tris-HCl at pH 7.5) was prepared and equal number of protoplasts from *H. koningii* and *T. asperellum* strains was mixed. To this 200 µl of 40% (w/v) polyethylene glycol (PEG, MW 6000; Sigma Chemicals Co., St. Louis, USA), 10 mM CaCl2 and 10 mM Tris-HCl, (pH 7.5) was added and gently mixed by rolling the tube. This step was repeated twice and the mixture was incubated at 28°C for 10 min with 1.1 ml of STC buffer by mixing gently. These dilution steps were repeated two times and 2.2 ml of STC was added. After the fusion and dilution, protoplasts were recovered by centrifugation at 10,000 rpm for 1 min and suspended in 5 ml STC. The interfused protoplasts were serially diluted in STC and plated on selective media.

Dual culture test

The antagonistic effects of each *Trichoderma* sp. and their fusants against *Pythium ultimum* was tested according to the method described by Fahmi *et al*., (2012). The percentage of inhibition (I %) on the mycelial growth of Pathogens were calculated using this formula: Percentage of inhibition (I %) = [(R1 - R2)/ R1] × 100 Where, R1 = radius of the pathogen away from the antagonist and R2 = radius of the pathogen.

SEM procedures

The parasitism of hyphal cells of grapevine pathogens by fusants was studied in detail by scanning electron microscopy (SEM) according to Asensio *et al*., (2005).

Results and Discussion

Morphological identification and Phylogeny of Trichoderma isolates

Cultures of *Trichoderma* aggregate species were morphologically differentiated on the bases of conidiophore branching pattern and conidium morphology according to the key developed by Rifai (1969) and Bissett (1991). The morphological identification of the *Trichoderma* was done and two isolates of *Hypocrea* and *Trichoderma* was selected for further study. Data from 5.8S-ITS region sequence is useful in establishing the coarse scale phylogeny of *Hypocrea* and *Trichoderma* for sorting them into statistically supported family-level groupings. We therefore used 5.8S-ITS sequence analysis to establish the origin of *Trichoderma* and *Hypocrea* isolates. An approximately 600 bp of 5.8S-ITS rDNA fragment was successfully amplified and sequenced from both parental isolates. Next, we performed a BLAST search with the *Trichoderma* and *Hypocrea* species 5.8S-ITS rDNA gene to find most similar sequences in GenBank. The BLAST of the obtained sequence was identified these isolates as *H. koningii* and *T. asperellum*, respectively. As can be seen from the Phylogenetic tree in Fig. 1 the two parental strains were almost 100% similarity with *Hypocrea koningii* and 99% similarity with *Trichoderma asperellum.* In our study, DNA sequencing of the 5.8S-ITS region of two parental isolates of protoplast fusion was carried out. The ITS region is one of the most reliable loci for the identification of a strain at the species level (Kullnig-Gradinger *et al.,* 2002). By comparing the sequences of the 5.8S-ITS region to the sequences deposited in GenBank database, all of the *Trichoderma* isolates can be identified to species level with homology percentage of at least 99%. However, Druzhinina and Kubicek (2004) mentioned that GenBank database contain many sequences of *Trichoderma* isolates and it was recently used by many literatures and resulted in successful identification of *Trichoderma* isolates (Anees *et al*., 2010 and Migheli *et al.*, 2009). From the *Trich*OKEY results obtained, all isolates were identified, and the results were in agreement with the BLAST results.

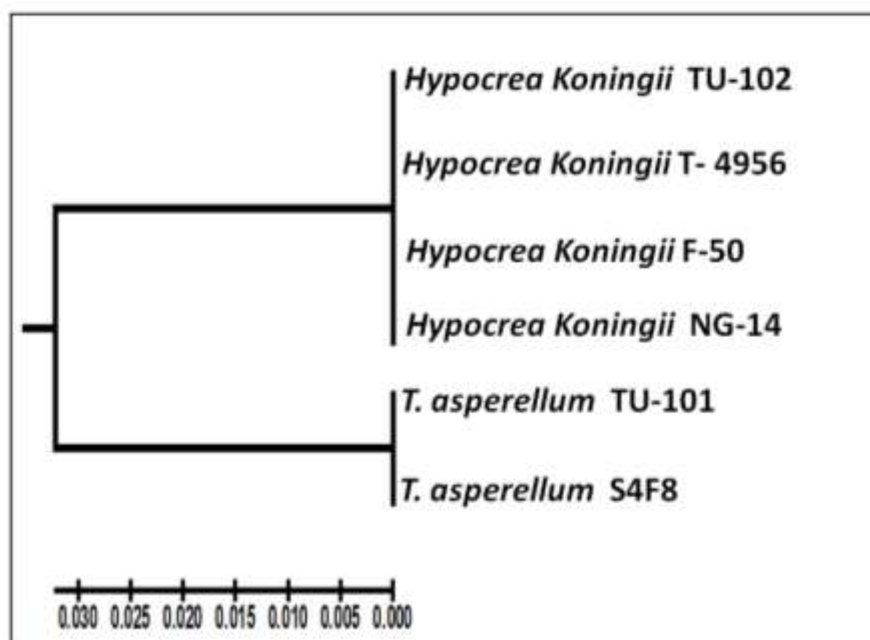

Fig. 1: Phylogenetic tree and the diversity of 5.8S-ITS region sequences of *Hypocrea* and *Trichoderma* strains

Isolation and protoplasts fusion

Incubation of *H. koningii* and *T. asperellum* mycelium with Novozym 234 resulted in lysis of cell wall and release of protoplasts. Swelling and rounding up of cell content were observed initially and subsequently the *H. koningii* and *T. asperellum* mycelium started lysing after 2 h. Almost complete digestion of mycelia and release of protoplasts occurred prominently after 3 h of incubation Fig. 2. The protoplasts just released out of mycelium were smaller in size but later they slowly enlarged to a spherical structure. When the protoplasts were mixed with PEG solution, they stuck together and pairs of protoplasts were observed. Later the plasma membranes in the place of contact of both the protoplasts dissolved and fusion of protoplasmic contents took place. Subsequently the nuclei of the pairing protoplasts fused together (karyogamy) in many cases and in some cases, dikaryotic stage without nuclear fusion was observed. Finally, the fused protoplasts became single, larger and round or oval shaped structures. Protoplast fusion is an effective tool for bringing genetic recombination and developing superior hybrid strains in filamentous fungi (Mrinalini and Lalithakumari 1998 ; Lalithakumari 2000). In this present study, demonstrated the interspecific crossing by protoplast fusion for genetic recombination in *Aspergillus niger* and *Trichoderma* Further, Hassan *et al*., (2011), achieved intra-specific hybridization in *T. harzianum*. The commercial Lysing enzymes (Sigma Chemicals Co.) at 8 mg ml^{-1} prepared in STC buffer was used to release the protoplasts from PTk-C and PTv-T with 0.6 M KCl as osmotic stabilizer. We have already optimized the conditions for releasing the protoplasts at our laboratory using different permutation combinations in various filamentous fungi including *Trichoderma* Hassan *et al*., (2011). Interestingly we observed that the release of protoplasts was significantly affected by the concentrations of Lysing enzymes. At low concentrations, the lysis of fungal mycelium took place only at the tip portion resulting in a minimum release of protoplasts whereas at high enzyme concentrations, though the mycelium effectively lysed, the protoplasts bursted immediately after release and disintegrated. Among different concentrations of Lysing enzymes tried, we optimized that 8 mg ml^{-1} with 0.6 M KCl as osmotic stabilizer to release higher number of protoplast from different *Trichoderma* spp. However, Pe'er and Chet (1990), obtained highest protoplasts from *T. harzianum* using Novozym 234 at 10 mg ml^{-1} with 0.6 M KCl and Tschen and Li (1997), used 15 mg ml^{-1} of Novozym with 0.6 M sucrose to isolate maximum protoplasts from *T. harzianum* and *T. koningii*. Further, Balasubramanian *et al.,* (2003), obtained maximum number of protoplasts from *Trichothecium roseum* using Novozym 234 in combination with chitinase and cellulase each at 5 mg ml^{-1}. Protoplasts fusion in *Trichoderma* has been achieved using 40 % PEG that was already reported as optimum concentration for interspecific fusion of protoplasts between *T. harzianum* and *T. longibrachiatum* Mrinalini and Lalithakumari (1998). However, Pe'er and Chet (1990) used 33 % PEG for intra-specific protoplast fusion in *T. harzianum*. The concentration of PEG is highly critical for effective fusion of protoplasts. Higher concentrations of PEG caused shrinking and bursting of protoplasts. The concentration between 40 and 60% was suitable for protoplasts fusion in different fungi. (Lalithakumari, 2000; Lalithakumari and Mathivanan 2003).

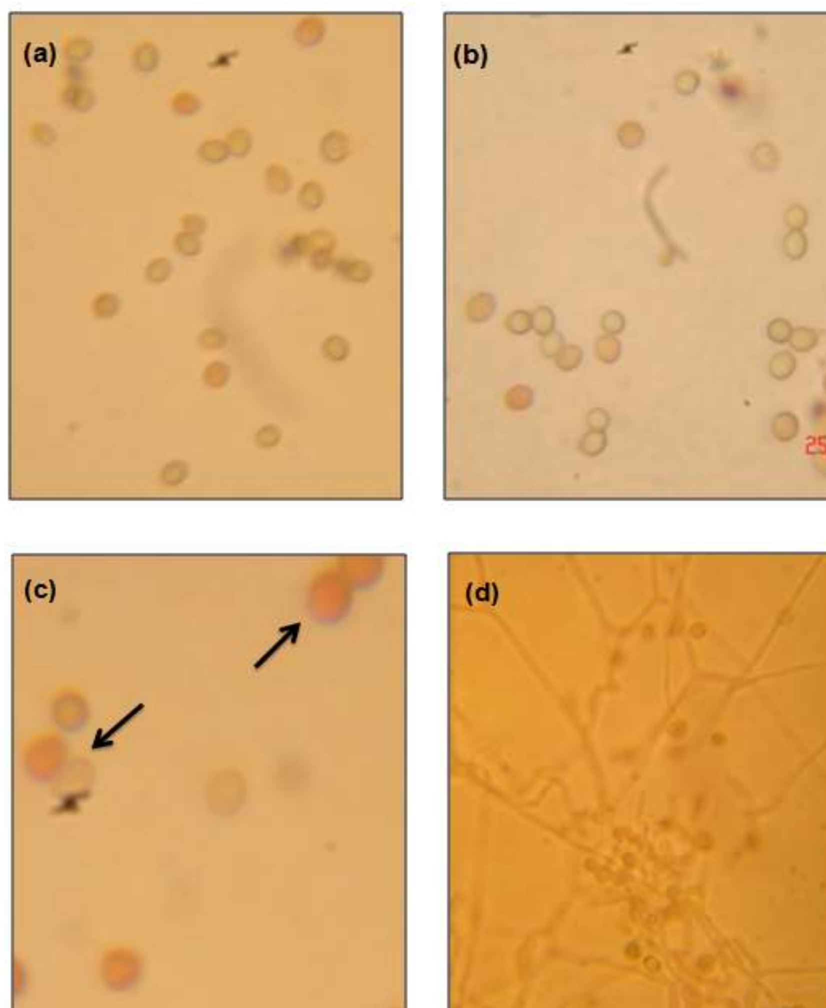

Fig. 2: Protopast fusion stages: Released protoplasts from parent strains observed after 3 h of incubation with lysis enzyme a and b, Fusion of protoplasts of *H. koningii* and *T. asperellum* after treatment with polyethylene glycol c, Regeneration of fused protoplasts

Antagonistic activity against some grapevine pathogens

Antagonistic effects of both *H. koningii and T. asperellum* and their fusant strains were tested against *Pythium ultimum* and *Fusarium roseum* on PDA at 28°C for 7 days. In all the dual culture plates tested, the contact zone was a curve, with concavity oriented towards the pathogenic fungi. The averaged inhibition percentage (I %) of mycelial growth for grapevine pathogens were presented in Fig. 3. All fused strains showed the ability to inhibit the mycelial growth of grapevine pathogens *P. ultimum and F. roseum.* However, Fus. 1 and Fus. 2 exhibited the lowest inhibition to the mycelial growth of *P. ultimum* with inhibition percentage of 72%, Fus. 4 exhibited the highest percentage of inhibition, 82%. Moreover, among all *Trichoderma* and *Hypocrea* fused strains, Fus. 6 exhibited the lowest inhibition to the mycelial growth of *Fusarium roseum* with inhibition percentage of 60% while Fus. 4 exhibited the highest percentage of inhibition 75%. SEM as show in Fig. 4, the parasitic hyphae reached the host hyphae and grew on the surface always with coiling and spores formation over grapevine pathogens *P. ultimum* and *F. roseum*. The antagonistic capacities of all *Trichoderma* fusant isolates and their parents against *P. ultimum* and *F. roseum* were tested using dual culture method. In all the dual culture plates, the contact zone appeared as a curve, with concavity oriented towards pathogens. The curvature of the contact area between the colony of antagonistic fungi and the colony of pathogenic fungi in the same PDA plate depend on the growth rate of the colonies. If one colony has a faster growth rate than the other, a curve in the contact zone will most probably be observed. However, if the two colonies have the same growth rate, a straight line would be observed when mycelia from both fungi come into contact (Hayat and Christias 2010; Petrescu *et al.,* 2012; El-Refai *et al.,* 2013). Moreover, all *Trichoderma* isolates exhibited inhibition to the mycelial growth of all pathogens. This could be due to

the production of diffusible components, such as lytic enzymes or water-soluble metabolites Anees *et al*., (2010).

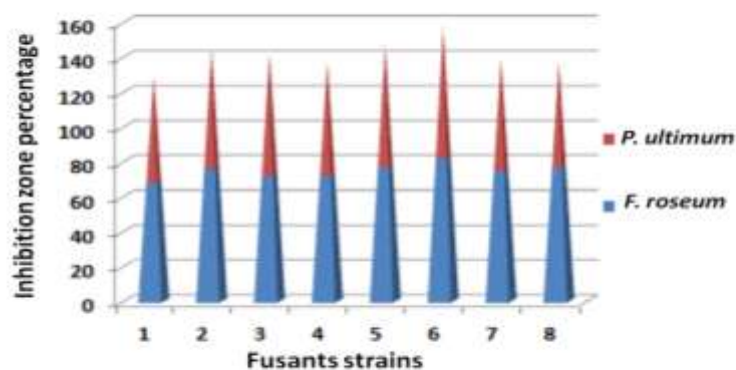

Fig. 3: Antagonistic activity of *Hypocrea* and *Trichoderma* and their fusants against *P. ultimum* and *F. roseum,* 1= *Hypocrea* and 2 = *Trichoderma*, 3-8 = their fusants.

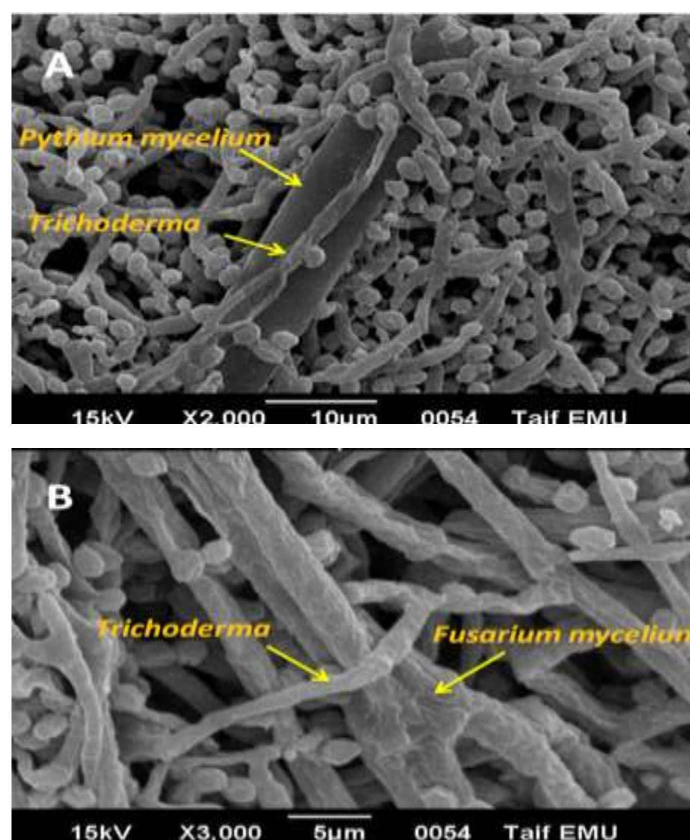

Fig. 4: Scanning electron micrograph micrographs showing antagonistic activity of Fus. 4 against *P. ultimum* and *F. roseum*

Refrences

Anees M, Tronsmo A, Hermann V, Hjeljord LG, Heraud C and Steinberg C (2010) Characterization of field strains of *Trichoderma* antagonistic against *Rhizoctonia solani*. *Fungal Biology*, **114**: 691-701. DOI 10.1016/j.funbio.2010.05.007

Asensio L, Lopez-Llorca LV, Lopez-Jimenez JA (2005) Use of light, scanning electron microscopy and bioassays to evaluate parasitism by entomopathogenic fungi of the red scale insect of palms (*Phoenicococcus marlatti*). *Micron*, **36**: 169–175. DOI: 10.1016/j.micron.2004.09.004

Balasubramanian N, Juliet A, Srikalaivani P and Lalithakumari D (2003) Release and regeneration of protoplasts from *Trichothecium roseum*. *Canadian Journal of Microbiology*, **49**: 263–268. DOI: 10.1139/w03-034

Bissett J (1991) A reveision of the genus *Trichoderma*. I. Intrageneric classification. *Canadian Journal of Botany*, **69**: 2357 - 2372. DOI: 10.1139/b91-297

Chaverr P, Castlebury LA, Overton BE and Samu GJ (2003) *Hypocrea/Trichoderma*: species with conidiophore elongations and green conidia. *Mycologia*, **95**: 1100-1140. DOI: 10.2307/3761915

Druzhinina I and Kubicek CP (2004) Species concept and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters. *Journal of Zhejiang University Science*, **6**:100-112.

El-Refai IM, Said AM, Assawah W and Draz MS (2013) Vegetative compatibility and strain improvement of some Egyptian *Trichoderma* isolates. *Life Science Journal*, **10**(3): 187-197.

Fahmi AI, Al-Talhi AD and Hassan MM (2012) Protoplast fusion enhances antagonistic activity in *Trichoderma spp*. *Nature and Science*, **10**(5): 100-106.

Feng X, Haojie J, Li H, Li T, Wang J, Jing L and Chen S (2011) Genome shuffling of *Trichoderma viride* for enhanced cellulase production. *Annual Microbiology*, **65**: 1-7.

Hassan MM, Al-Awady M A, Lakhani H N and El-Tarras AE (2013) Improvement of biological control activity in *Trichoderma* against some grapevine pathogens using protoplast fusion. *Life Science Journal*, **10**(4): 2275-2283.

Hassan MM, Eissa RA and Hamza HA (2011) Molecular characterization of intraspecific protoplast fusion in *Trichoderma harzianum*. *New York Science Journal*, **4**: 48-53.

Hayat S and Christias C (2010) Isolation and fusion of protoplasts from the hytopathogeneic fungus *Sclerotium rolfsiiL* (SACC). *Brazilian Journal of Microbiology*, **41**: 253-263. DOI: 10.1590/S1517-83822010000100035

Hermosa MR, Grondona I, Iturriaga EA, Diaz-Minguez JM, Castro C and Monte E, (2000) Molecular characterization and identification of biocontrol isolates of *Trichoderma spp*. *Applied Environmental Microbiology*, **66**: 1890-1898. DOI: 10.1128/AEM.66.5.1890-1898.2000

Kullnig-Gradinger CM, Szakacs G and Kubicek CP (2002) Phylogeny and evolution of the genus *Trichoderma*: a multigene approach. *Mycology Research*, **106**: 757-767. DOI: 10.1017/S0953756202006172

Lalithakumari D (2000) Fungal protoplasts a biotechnological tool, Oxford & IBH *Publishing Company Private Limited*, New Delhi, India.

Migheli Q, Balmas V, Zelazowska M, Scherm B, Fiori S, Kopchinskiy AG, Kubicek CP and Druzhinina ID (2009)

Soils of a Meditteranean hot spot of biodiversity and endemism (Sardinia, Tyrrhenian Islands) are inhabited by pan-European, invasive species of *Hypocrea/Trichoderma*. *Environmental Microbiology*, **11**: 35-46. DOI: 10.1111/j.1462-2920.2008.01736.x

Mrinalini C and Lalithakumari D (1998) Integration of enhanced biocontrol efficacy and fungicide tolerance in *Trichoderma* spp. by electrofusion, *Journal Plant Disease Protection*, **105**: 34–40.

Pe′er S, and Chet I (1990) *Trichoderma* protoplast fusion; a tool for improving biocontrol agents, *Canadian Journal Microbiology*, **36**: 6–9.

Petrescu E, Sesan T and Oprea T (2012) *In vitro* evaluation of the relationships between some fungal pathogens of black currant crop and some saprophytic fungi. *Scientific Bulletin.* **16**: 175-178.

Rifai MA (1969) A revision of the genus *Trichoderma*, *Mycological Papers*, **116**: 1 - 16.

Salama SA and Tolba AF (2003) Identification of *Trichoderma reesei* using specific molecular marker and their ability as biocontrol agent. *Egyptian Journal of Genetics Cytology*, **32**: 137-151.

Stasz TE, Harman GE and Weeden NF (1988) Protoplast preparation and fusion in two biocontrol strains of *Trichoderma harzianum*. *Mycologia*, **80**: 141–150. DOI: 10.2307/3807788

Tschen JS and Li IF (1997) Optimization of formation and regeneration of protoplasts from biocontrol agents of *Trichoderma* sp. *Mycoscience*, **35**: 257–263. DOI: 10.1007/BF02268447