# *In vitro* ASSESSMENT OF *Trichoderma asperellum* ISOLATED FROM PLANT RHIZOSPHERE AND EVALUATION OF THEIR POTENTIAL ACTIVITY AGAINST SOME PATHOGENIC FUNGI

**G. M. HASSAN AND NADA F. HEMEDA**

*Department of genetics, Faculty of Agriculture, Fayoum University, 63514 Egypt*

Fungal pathogens can cause dangerous and invasive diseases in economic plants in the world. The pathogenic fungi, in particular, soil borne pathogens act a significant agent in the important losses of agricultural resources in the developing world. Accurate and definitive fungal identification are essential for correct disease diagnosis and treatment of associated with fungal infections. Biological control of plant pathogens using *Trichoderma* species becomes a natural friendly ecological approach to overcome hazards associated with chemical fungicides which cause of many environmental pollutions and development of resistant strains (Widyastuti *et al*., 2003). Antagonistic strains of *Trichoderma* have indirect and direct mechanisms for inhibition growth of plant pathogenic fungi and improve the overall health of plants, indirectly by competing for nutrients and space. The indirect and direct mechanisms may act coordinately, and their importance in the biocontrol process depends on the *Trichoderma* spp., the crop plant and the environmental conditions (Benitez *et al*., 2004). The antagonistic and mycoparasitic potential of the *T. asperellum* strains were evaluated in *vitro* and *in vivo*, and they showed that all *T. asperellum* strains were antagonistic to *Pythium myriotylum* by Mbarga *et al*. (2012). The antagonistic activity of 30 isolates of *T. asperellum* against four different isolates of *Fusarium oxysporum* was evaluated. All the isolates of *T. asperellum* significantly reduced the mycelial growth of *Fusarium* isolates with a significant variation in the growth reduction of *Fusarium* isolates depending on the activity of *T. asperellum* isolates. The ability of *Trichoderma* to parasitize and kill other fungi has been the major driving force behind the commercial success of these fungi as bio-fungicides (Segarra *et al*., 2010). The genome size of mycoparasites of *Trichoderma* species ranged from 36.1 Mb to 40.98 Mb. The genome size of *T. asperellum* is 37.4 Mb (Druzhinina *et al*., 2011). The results of many previous studies were summarized and they documented that *T. asperellum* and *T. asperelloides* are highly rhizosphere competent and are able to stimulate growth and immune defense of plants. *Trichoderma viride* as a biological control agent was inoculated into the soil, to suppress the activity of the pathogenic fungi *Fusarium oxysporum* and *Rhizoctonia solani*on in tomato (Harman

*Egypt. J. Genet. Cytol., 45: 113-128, January, 2016*
Web Site (*www.esg.net.eg*)

*et al.*, 2004). *Trichoderma* species were characterized and distinguished by morphological characteristics and provided detailed observations on the morphological characters of defined species in *Trichoderma.* Taxonomy of *Trichoderma* is based on morphological characteristics alone for detecting *Trichoderma* has not led to a satisfactory taxonomy between *Trichoderma* species (Samuels *et al*., 2004). In contrast, molecular techniques such as PCR and DNA sequencing are very sensitive, reliable and rapid methods for species detection. Molecular markers demonstrate the variation in DNA sequences within and between the species and provide the basis for precise identification. Polymerase chain reaction (PCR) methods have found widespread use for pathogen identification, and a number of PCR-based assays have been developed for use in the diagnosis and characterization of *Trichoderma* species (Jaklitsch, 2009). The rRNA is essential for the survival of all cells and the genes encoding the rRNA are highly conserved in the fungal and other kingdoms. The universal primers were used for amplification of the 18S rRNA gene fragment and strain was characterized by using 18S rRNA gene sequence. The identified strain *T. atroviride* TAU8 based on phylogenetic tree analysis together with the 18S rRNA gene sequence. The sequences of the rRNA and proteins comprising the ribosome are highly conserved throughout evolution because they require complex inter and intra-molecular interactions to maintain the protein synthesizing machinery (Singh *et al*., 2014). Molecular techniques such as RAPD and rRNA sequencing have been used widely for taxonomic conclusions in various organisms including *Trichoderma* spp. Therefore, it is necessary to correlate molecular phylogeny with morphological and other biochemical and physiological traits (Kim *et al*., 2000). In eukaryotic cells, rRNA cistrons made up of 18S, 5.8S and 28S rRNA genes are transcribed by RNA polymerase I. Then, RNA splicing of the cistrons will remove the two internal transcribed spacers flanking the 5.8S gene. The two spacers, together with the 5.8S gene, are normally referred to as the ITS region (Schoch *et al*., 2012). The rRNA genes are universally conserved while the ITS region and intergenic spacer (IGS) are highly variable. The ITS region and IGS region are the fastest evolving regions and they may vary among species within a genus. Thus, the sequences of these regions can be used for identification of closely related species (Lieckfeldt *et al*., 2002; White *et al*., 1990). However, understanding the genetic variability within *Trichoderma* strains and their biological and biochemical activities are necessary to improve the selection of the different isolates as biocontrol agents (Consolo *et al*., 2012). To the best of our knowledge, there is no information on the occurrence of *T. asperellum* in Egypt and this is the first report of the occurrence and isolation of *T. asperellum* from Egyptian soils. The present study aims to (1) isolate and identify new biofungicides from Egyptian soils using morphological and molecular characters, (2) assess the potential antagonistic activity of *Trichoderma* isolates against *Fusarium*

*semitectum* and *Alternaria alternata* (3) Provide new bio-fungicides for Egyptian researchers to control of some plant pathogens (*Fusarium* and *Alternaria*) and to overcome hazards associated with chemical fungicides which cause of many environmental pollutions and development of resistant strains reduce the risk of the use of synthetic fungicides.

## MATERIALS AND METHODS

### *Collection of rhizosphere samples*

Soil samples were collected from rhizosphere of different crops includes *Vicia faba*, *Vigna unguiculata* and *Phaseolus vulgaris* grown in agricultural fields, Fayoum governorate, Egypt. From each crop, the soil particles tightly adhered with root surface were removed separately and collected in sterile polyethylene bags, which were transported to the laboratory and stored at 4°C until use.

### *Isolation of Trichoderma from collected samples*

The genus of *Trichoderma* isolates were isolated from rhizosphere samples according to soil dilution plate method described by Kucuk and Kivanc (2003), with some modifications. One ml of each appropriate dilution ($10^{-3}$ to $10^{-5}$) was pipetted in petri dishes. Sterilized Rose Bengal Agar medium (RBA) was cooled to 45°C and then poured and left to solidify. All plates were incubated at 28°C for 7 days. The culture plates were examined daily and individual colonies were isolated and purified, then transferred to fresh potato dextrose Agar medium (PDA). Distinct morphological characteristics were observed for identification and the plates were stored at 4°C for further experiments.

### *Morphological characterization and microscopic study of Trichoderma isolates*

Morphological characterizations, including (mycelial color, colony texture and shape) and microscopic observations, including (conidia shape, conidia color, conidiophore - branching, phialide width and phialide length) were conducted according to Sharma and Singh (2014). Considering all the morphological characters, isolates of *Trichoderma* were placed under suitable group according to an interactive key provided by Samuels *et al*. (2002) at http://nt.arsgrin.gov/taxadescriptions/keys/FrameKey.cfm? gen= Trichoderma.

### *Screening of Trichoderma isolates for their chitinase production*

Colloidal chitin was prepared according to the method used in our laboratory (Hassan *et al*., 2015). Based on the morphological characterization and microscopic study, isolates Tas 1, Tas 2, Tas 3 and Tas 4 were screened for chitinase production by using the method of Agrawal and Kotasthane (2012). Chitinase detection medium contained the following components (g/litter): colloidal chitin (4.5 g), $MgSO_4.7H_2O$ (0.3 g), $(NH_4)_2SO_4$ (3.0 g), $KH_2PO_4$ (2.0 g), citric acid monohydrate (1 g), agar (15 g), bromocresol purple (0.15 g) and 200 µl of tween-80. The agar medium was autoclaved at 121°C for

15 min. then cooled to 45°C and the pH was adjusted to 4.7 by 1N sterile HCL. Fresh culture plugs of the *Trichoderma* isolates were placed in the middle of the agar plates and incubated at 28°C for 7 days. Chitinase activity exhibited by *Trichoderma* isolates was determined by measuring the diameter of the purple color zone after 7 days incubation.

### *In vitro antagonistic effect of Trichoderma isolates against plant pathogen fungi*

Pathogenic fungi (*Fusarium semitectum* and *Alternaria alternata*) were kindly donated by Plant pathology department, Faculty of Agriculture, Fayoum University, Egypt. *T. asperellum* isolates were evaluated for their potential to antagonize the plant pathogenic fungus of *A. alternata* and *F. semitectum in vitro* using a dual culture technique according to the bioassay method described by Zhang and Wang (2012). Petri dishes with the PDA medium were inoculated with discs five millimeters in diameter of the six-day old culture of selected *Trichoderma* isolates and the pathogenic fungi (*A. alternata* and *F. semitectum).*The discs of *Trichoderma* isolates and pathogenic fungi were placed on the opposite sides of each petri dish. The dual culture was incubated at 28°C for 7 days and the zone of inhibition was measured. Three replicates were used in each test and for each *Trichoderma* isolates, the percentage inhibition of the average radial growth was calculated in relation to the growth of the control according to Mokhtar and Aid (2013) as the following: L= (C-T/C) X 100. Where L: percentage inhibition in the growth of the pathogen, C: radius (cm) of the pathogen colony in control culture, T: radius (cm) of the pathogenic colony in the treatment.

### *Genomic DNA isolation from selected Trichoderma isolates*

Mycelium of *Trichoderma* isolates collected from 7 days old culture plates and 250 mg mycelium were added to 2 ml tubes and used to extract DNA using theWizard® Genomic DNA Purification Kit (Promega, USA).

### *PCR amplification of rDNA region of Trichoderma isolates*

PCR was performed using two universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG -3') for forward primer and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') for the reverse primer to amplify the Internal Transcribed Spacer1 (ITS) regions between the small and large nuclear rDNA region of selected *Trichoderma* isolates (Tas1, Tas2, Tas3 and Tas4). Sequences of primers were commercially designed according to White *et al*. (1990). PCR reactions were performed in a total volume of 50 µl, containing 100 ng of the template DNA, 0.2 µM concentration of each primer, 200 µM concentration of each dNTPs and 2.5U of Taqpolymerse enzyme in 10X PCR buffer (100mM Tris-HCl, pH 8.3, 500 mMKCl). PCR amplification was performed in a thermal cycler 2720 (Applied Biosystems, USA).The amplification program consisted of an initial denatura-

tion step at 95°C for 5min, 35 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 55°C, 2 min extension at 72°C, and a final extension for 10 min at 72°C. Expected molecular size (600 bp) of PCR products were checked by electrophoresis on 2% agarose gel and stained with ethidium bromide for visualization under UV light. The amplified PCR product was purified using Montage PCR Clean up Kit (Millipore), following manufacture instructions to remove unincorporated PCR primers.

***Sequencing of ITS region of Trichoderma isolates***

The purified PCR products of approximately 600 bp were subjected to sequencing through lab technology services located in Korea and performed at Applied Biosystems model 3730XL automated DNA sequencing system.

***Computational analysis (Blast) and construction of phylogenetic tree***

The data of the nucleotide sequence of the ITS regions of r RNA obtained from the four *Trichoderma* isolates were compared with ITS sequences collected from *Trichoderma* sequences available in GenBank database (http://www.ncbi.nlm.nih.gov/ Blast). The nucleotide sequences were aligned using Clustal W multiple sequence alignment (Thompson *et al*., 1994). The phylogenetic dendrogram was constructed by the neighbour-joining method using MEGA version 5 software program (Molecular Evolutionary Genetic Analysis) according to the method of Tamura *et al*. (2011).

## RESULTS AND DISCUSSION

***Isolation and morphological identification of Trichoderma isolates***

Fifteen *Trichoderma* isolates were isolated from samples collected from rhizosphere soil of different cultivation crops. Genus and species level identification of *Trichoderma* isolates were done based on morphological and microscopic observation. The confirmation of species-level identification of *Trichoderma* isolates was carried out according to an interactive key provided by Samuels *et al*. (2002) at http://nt.ars-grin.gov/ taxadescriptions/keys/FrameKey.cfm? gen-Trichoderma. Four out of fifteen *Trichoderma* isolates were belonging to *T. asperellum* strains according to their morphological characters and an interactive key. All four *T. asperellum* grow rapidly on PDA forming a smooth surfaced, watery white mycelia mat which soon becomes whitish green and then dull green with the production of conidia. The isolates possessed repeatedly branched conidiophore, smooth central axis from which secondary branches arise and tend to be paired. The size of conidia and some morphological characteristics of the selected *T. asperellum* isolates are summarized in Table (1). Colony appearance of the four *T. asperellum* isolates grows on PDA for 7 days at 28°C was shown in Fig. (1). The colony of four *T. asperellum* isolates (Tas1, Tas2, Tas3 and Tas4) showed grew moderately forming rings of dense

conidial production, hyphae lawn, mycelial sparse and grew close to the agar aerial mycelium, conidiophore regularly branched and typically paired, phialide straight, conidia L/W 1.1-1.2 µm, green to dark green. Chlamydospore typically abundant within one week, terminal or infrequently intercalary, hyphae, subglobose, terminal, smooth and pale green. No distinct pigments were detected (Table 1 and Fig. 2). Morphological characterization was conventionally used in the identification of *Trichoderma* species, and it remains as a potential method to characterize and distinguish *Trichoderma* species (Samuels *et al*., 2002; Anees *et al*., 2010). The few morphological characters with limited variation may lead to an overlap and misidentification of the strains and showed the necessary of DNA-based characters to complete identification evident from the present study.

***Screening of selected Trichoderma asperellum isolates for chitinase production and their activity***

Primary screening of chitinase producing *Trichoderma* is essential to obtain an efficient biocontrol agent. Each of the four *T. asperellum* isolates was screened for chitinolytic enzyme production based upon the color intensity and the diameter of the purple colored zone surrounding the colony on chitinase detection medium in shorter time. The results of chitinase production were presented in Fig. (3). Purple color zone was due to bromocresol purple that was supplemented with media as a pH indicator. As the medium containing colloidal chitin, *Trichoderma* breaks down the chitin with the chitinolytic enzymes, changing chitin to N-acetylglucosamine, which is fundamental in nature. Thus, change in the pH from acidic to basic, color of media also changes from yellow to purple color (Kotasthane and Agrawal, 2009). Chitin agar plate has been used earlier for isolating chitinolytic microorganisms by observing clear zone around the colony of microorganism (Wirth and Wolf, 1990).

***In vitro antagonistic efficacy* of *Trichoderma asperellum isolates against plant pathogenic fungi***

The antagonistic effect of the four *T. asperellum* isolates was observed against *F. semitectum* and *A. alternata* in a dual culture growing on PDA at 28°C after 7 days. The inhibitions of mycelial growth of pathogenic fungi were shown in Fig. (4). In all the dual culture plates tested, the contact zone was a curve, with the concavity oriented towards the pathogenic fungi. In the negative control plates, only pathogenic fungi were inoculated (Fig. 4). The results represented in Fig. (5) showed that all selected *T. asperellum* isolates exhibited inhibition on the mycelial growth of *A. alternata* and *F. semitectum*. The highest inhibition percentage against *A. alternata* was recorded for *Trichoderma* isolate Tas 4 (69.23%), followed by Tas3, Tas1 and Tas2 which were 63.07, 60.0 and 58.46%, respectively, while, the highest inhibition percentage against *F. semitectum* was recorded for Tas1 (64%), followed by Tas2, Tas4 and

Tas3 which were 61.33, 60.0 and 58.66%, respectively. The potentiality of *Trichoderma* spp. as biocontrol agents of phytopathogenic fungi in several crops are well known especially to *Fusarium* spp. and *Rhizoctonia* spp. (Poddar *et al.*, 2004; Rojo *et al.*, 2007). *Trichoderma* suppressed the growth of *F. semitectum* through the overgrowth. In another case, *Trichoderma* was observed to cluster around *F. semitectum* by the formation of small tufts thus limiting the growth of the pathogen of sheath blight. The formation of sclerotial bodies of *F. semitectum* was suppressed (Shalini and Kotasthane, 2007). Tondje *et al.* (2007) reported that direct mycoparasitism is considered to be the main mechanism of action for *T. asperellum*. The curvature of the contact area between the colony of antagonistic fungi and the colony of pathogenic fungi in the same PDA medium depends 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, the average inhibition percentage of mycelial growth of pathogenic fungi was frequently used and shown to be a useful way in assessing the antagonistic potential of the antagonistic fungi. Overall the four *T. asperellum* isolated in this study have the ability to inhibit the mycelial growth of *A. alternata* and *F. semitectum* (Petrescu *et al*., 2012). This result was in agreement with the results found by Ommati and Zaker (2012), they found that *T. asperellum* had the highest inhibition of the growth of *F. oxysporum*. *T. asperellum* was used as an efficient biological control agent in controlling *Fusarium* wilt in tomato (Segarra *et al*., 2010). The rapid growth of *Trichoderma* is an important advantage in competition with plant pathogenic fungi for space and nutrients (Deacon and Berry, 1992).

### *PCR amplification of ITS region of rDNA of Trichoderma asperellum isolates*

Genomic DNA of the four selected *T. asperelllum* isolates was analyzed by PCR amplification of rDNA gene including 5.8S gene and the flanking intergenic transcribed spacer ITS region of rDNA. Amplification of the ITS with primers ITS1 and ITS4 yielded a single product estimated by gel electrophoresis of approximately 600 bp from all the four PCR amplification produced from isolates of *T. asperellum*. In this study, the morphological identification of *Trichoderma* isolated from Egyptian rhizosphere soils was complemented by a molecular identification based on internal transcript spacers (ITS region) of rDNA sequences. Based on the phenotypic characteristics (colony texture, conidia, phialides, chlamydospore and conidiophore) and genotypic characteristics (ITS region of 18S rRNA), Tas1, Tas2, Tas3 and Tas4 were identified as *T. asperellum*. These results indicate that molecular systematic studies based on the sequence of ITS region are important for confirmation of phenotypic characterization of *Trichoderma* isolates. The rRNA is essential for the survival of all the cells

and the genes encoding rRNA is highly conserved in the fungal. In eukaryotes, the genes encoding ribosomal RNA are organized in arrays which contain repetitive transcriptional units involving 16-18S, 5.8S and 23-18S rRNA, two transcribed intergenic spacers ITS1 and ITS2. These units are transcribed by RNA polymerase 1 and separated by non- transcribed intergenic spacer (IGS). The product of RNA polymerase 1 is processed in the nucleolus, where the ITS 1 and ITS2 are excised and three types of rRNA are produced (Shahid *et al*., 2014). The ITS sequence was chosen for this analysis because it has been shown to be more informative with various sections of the genus *Trichoderma* (Kuhls *et al*., 1997; Ospina-Giraldo *et al*., 1998).

***Sequencing of ITS region of Trichoderma asperellum isolates and Phylogenetic analysis***

The sequence of the four selected *T. asperellum* isolates was done to confirm species identified previously, according to their morphological and microscopic observation. PCR products amplified from the four *T. asperellum* isolates were sequenced. They could be aligned and a consensus sequence was generated from each alignment made. Then, BLAST was used to determine the species identity of *Trichoderma* isolates. The sequences of ITS region of the four *T. asperellum* isolates were submitted to GenBank under the following accession numbers (KC898190, KC898191, KC898192 and KC898193) for the *T. asperellum* isolates (Tas1, Tas2, Tas3 and Tas4), respectively. The Phylogenetic tree obtained by sequence analysis of ITS1 and ITS4 of our *T. asperellum* isolates and the sequences of 25 *Trichoderma* spp obtained from NCBI, GenBank is represented in Fig. (6). According to the NCBI BLAST search of the sequence of our *T. asperellum* isolates against the sequences of 25 other *Trichoderma* spp. *Trichoderma* isolates (Tas1, Tas2, Tas3 and Tas4) were identified as *T. asperellum* (Fig. 6).

To the best of our knowledge, this is the first report on the occurrence and isolation of *T. asperellum* from Egyptian soils. The main reasons of this limitation may be due to most of the studies, depending on the morphological characterization key of Rifai (1969) for identifying the *Trichoderma* spp. In conclusion, our results suggest that molecular identification is very important to identify the *Trichoderma* species and it must be used to confirm morphological approaches in the identification of *Trichoderma* isolates. Each of the two methods has owned strengths and limitations. Accordingly, we must use combine morphological and molecular methods for successful identification of *Trichoderma* isolates. Worth mentioning that, the four *T. asperellum* isolates (Tas1, Tas2, Tas3 and Tas4) were high producers for chitinase and showed high antagonistic activity against the tested pathogenic fungi. However, further study must be done for developing these isolate as a new bio-fungicides at large scale production.

## SUMMARY

Hydrolytic enzymes producing *Trichoderma* species have long been recognized as an agent for controlling plant diseases caused by various phytopathogenic fungi. This study aims to isolate and characterize of new bio fungicides from Egyptian soils and assess of their antagonistic activity against some pathogenic fungi (*Fusarium semitectum* and *Alternaria alternata*). Four isolates of the *Trichoderma asperellum* were isolated from rhizosphere soil of different host plants collected from Fayoum governorate, Egypt. The isolates were characterized according to morphological characterization, microscopic observations and confirmed by sequencing of the ITS region of 18S rRNA. *Trichoderma asperellum* isolates were evaluated for their potential to antagonize the plant pathogenic fungi (*F. semitectum* and *A. alternata*) *in vitro* using the dual culture technique. Four out of twenty *Trichoderma* isolates (20%) were identified as *T. asperellum* based on morphological characteristics and confirmed by sequencing of ITS region of 18SrRNA. The four selected *T. asperellum* isolates (Tas1, Tas2, Tas3 and Tas4) were screened for their ability to produce chitinase on solid agar medium using bromocresol purple for developing the clear zone around colonies, and characterized due to its antagonistic effect against mycelial growth of pathogenic fungi. These results indicate that molecular systematic studies based on the sequence of ITS region are important for confirmation of phenotypic characterization of *Trichoderma* isolates. To the best of our knowledge, there is no information on the occurrence of *T. asperellum* in Egypt and this is the first report of the occurrence and isolation of *T. asperellum* from Egyptian soils.



## ACKNOWLEDGMENT

The authors are grateful to Mr. Sameh A. El-Naggar, an associate demonstrator in plant pathology department, Faculty of Agriculture, Fayoum University for help and assistance in the microscopic characterization of *Trichoderma* isolates.


## REFERENCES


Agrawal, T. and A. S. Kotasthane (2012). Chitinolytic assay of indigenous *Trichoderma* isolates collected from different geographical locations of Chhattisgarh in Central India. Springer Plus, 1: 73-82.

Anees, M., A. Tronsmo, V. Edel-Hermann, G. L. Hjeljord, C. Heraud and C. Steinberg (2010). Characterization of field isolates of *Trichoderma* antagonistic against *Rhizoctonia solani*. Fungal Biol., 114: 691-701.

Benitez, T., M. A. Rincon, M. C. Limon and C. A. Codon (2004). Biocontrol mechanisms of *Trichoderma* strains. Int. Microbial., 7: 249-260.

Consolo, V. F., C. I. Monaco, C. A. Cordo and G. L. Salerno (2012). Characterization of novel *Trichoderma* spp. isolates as a search for effective bio controllers of fungal diseases of economically important crops in Argentina. World J. Microbiol. Biotechnol., 28: 1389-1398.

Deacon, J. W. and L. A. Berry (1992). Modes of actions of mycoparasites in relation to biocontrol of soilborne plant pathogens. In: *Biological control of plant diseases*, eds. by E. C. Tjamos, G. C. Papavizas and R. J. Cook, p. 157-167. Plenum Press, New York.

Druzhinina, I. S., V. Seidl-Seiboth, A. Herrera-Estrella, B. A. Horwitz, C. M. Kenerley and E. Monte (2011). *Trichoderma* the genomics of opportunistic success. Nat. Rev. Microbiol., 9: 749-759.

Harman, G. E., C. R. Howell, A. Viterbo, I. Chet and M. Lorito (2004). *Trichoderma* species- opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol., 2: 43-56.

Hassan, G. M., Z. A. El-Feky, N. F. Hemad and M. A. Sayed (2015). Isolation and molecular characterization of Egyptian *Trichoderma* and assessment of their antagonistic potential against *Rhizoctonia*. J. Microb. Biotechnol. and Food Sci., 4: 495-502.

Jaklitsch, W. M. (2009). European species of *Hypocrea* Part I. The green spored species. Stud. Mycol., 63: 1-91.

Kim, K. Y., G. J. Lee, M. G. Ha, T. H. Lee and J. D. Lee (2000). Intrageneric relationships of *Trichoderma* based on internal transcribed spacers and 5.8S rRNA nucleotide sequences. Mycobiol., 28: 11-16.

Kucuk, C. and M. Kivanc (2003). Isolation of *Trichoderma* spp. and determination of their antifungal, biochemical and physiological features. Turk. J. Biol., 27: 247-253.

Kuhls, K., E. Lieckfeldt, G. J. Samuels, T. Borner, W. Meyer C. P. Kubicek (1997). Revision of *Trichoderma sect*. *Longibrachiatum* including related teleomorphs based on analysis of ribosomal DNA internal transcribed spacer sequences. Mycologia., 89: 442-460.

Lieckfeldt, E., K. Kuhls and S. Muthumeenakshi (2002). Molecular taxonomy of *Trichoderma* and *Gliocladium* and their teleomorphs, In: *Trichoderma* and *Gliocladium*: basic biology, taxonomy and genetics, eds. by C. P. Kubicek, and G. E. Harman, p. 35-53. Taylor & Francis Ltd.

Mbarga, J. B., G. M. Ten Hoopen, J. Kuate, A. Adiobo, E. L. Ngonkeu and Z. Ambang (2012). *Trichoderma*

*asperellum*: A potential biocontrol agent for *Pythium myriotylum*, causal agent of cocoyam (*Xanthosoma sagittifolium*) root rot disease in Cameroon. Crop Protect., 36: 18-22.

Mokhtar, H. and D. Aid (2013). Contribution in isolation and identification of some pathogenic fungi from wheat seeds, and evaluation of antagonistic capability of *Trichoderma harzianum* against those isolated fungi *in vitro*. Agric. Biol. J. N. Am., 4: 145-154.

Ommati, F. and M. Zaker (2012). Evaluation of some *Trichoderma* isolates for biological control of potato wilt disease (*Fusarium oxysporum*) under lab. and greenhouse conditions. Crop protect., 1: 279-286.

Ospina-Giraldo, M. D., D. J. Royse, M. R. Thon, X. Chen and C. P. Romaine (1998). Phylogenetic relationships of *Trichoderma harzianum* causing mushroom green mold in Europe and North America to other species of *Trichoderma* from world-wide sources. Mycologia, 90: 76-81.

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

Poddar, R. K., D. V. Singh and S. C. Dubey (2004). Integrated application of *Trichoderma harzianum* mutants and carbendazim to manage chickpea wilt (*Fusarium oxysporum*f. sp. *ciceri*). Indian J. Agric. Sci., 74: 346-348.

Rifai, M. (1969). A revision of genus *Trichoderma*. Mycological Papers, 116: 1-56.

Rojo, F. G., M. M. Reynoso, M. F. Sofia, N. Chulze and A. M. Torres (2007). Biological control by *Trichoderma* species of *Fusarium solani* causing peanut brown root rot under field conditions. Crop Protect., 26: 549-555.

Samuels, G. J., P. Chaverr, D. F. Farr and E. B. McCray (2002). *Trichoderma* Online. Systematic Mycology and Microbiology Laboratory, ARS, USDA; http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm.

Samuels, G. J., P. Chaverri, D. F. Farr and E. B. McCray (2004). *Trichoderma* Online, Systematic Botany & Mycology Laboratory, ARS, USDA. From http://nt.ars-grin.gov/taxadescriptions/keys/*Trichoderma* Index.cfm.

Schoch, C. L., K. A. Seifert, S. Huhndorf, V. Robert, J. L. Spouge and C. A. Levesque (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi, Proceed-

ing of the National Academy of Sciences of the USA, 6241-6246.

Segarra, G., E. Casanova, M. Aviles and I. Trillas (2010). *Trichoderma apserellum* strain T34 controls *Fusarium* wilt disease in tomato plants in soilless culture through competition for iron. Microb. Ecol., 59: 141-149.

Shahid, M., M. Srivastaval, V. Kumar, A. Singh, A. Sharma and S. Pandey (2014). Phylogenetic diversity analysis of *Trichoderma* species based on internal transcribed spacer (ITS) marker. Afr. J. Biotechnol., 13: 449-455.

Shalini, S. and A. S. Kotasthane (2007). Parasitism of *Rhizoctonia Solani* by strains of *Trichoderma* spp. EJEAF. Chem., 6: 2272-2281.

Sharma, K. K. and U. S. Singh (2014). Cultural and morphological characterization of rhizospheric isolates of fungal antagonist *Trichoderma*. J. Appl. and Nat. Sci., 6: 451-456.

Singh, A., M. Shahid and M. F. Srivastava (2014). Identification of *Trichoderma atroviride* TAU8/7445 strain by sequencing of 18S rRNA gene. Indian J. Agric. Biochem., 27: 85-87.

Tamura, K., D. Peterson, N. Petreson, G. Stechsr, M. Nei and S. Kumar (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., 28: 2731-2739.

Thompson, J. D., D. J. Higgins and T. J. Gibson (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positive specific gap penalties and weight matrix choice. Nucleic Acids Res., 22: 4673-4680.

Tondje, P. R., D. P. Roberts, M. C. Bon, T. Widmer, G. J. Samuels and A. Ismaiel (2007). Isolation and identification of mycoparasitic isolates of *Trichoderma asperellum* with potential for suppression of black pod disease of cacao in Cameroon. Biol. Control., 43: 202-212.

White, T. J., T. Bruns, S. Lee and J. Taylor (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, PCR Protocols: A Guide to Methods and Applications, 315-322.

Widyastuti, S. M., H. Sumardi and D. Yuniarti (2003). Biological control of *Scleorotium rolfsii* damping-off of tropical pine (*Pinus merkusii*) with three isolates of *Trichoderma* spp. Online J. Biol. Sci., 3: 95-102.

Wirth, S. J. and G. A. Wolf (1990). Dye-labelled substrates for the assay

and detection of chitinase and lysozymes activity. J. Microbiol. Meth., 12: 197-205.

Zhang, R. and D. Wang (2012). *Trichoderma* spp. from rhizosphere soil and their antagonistic against *Fusarium sambucinum*. Afr. J. Biotechnol., 18: 4180-4186.

Table (1): Some morphological and microscopic characters of *Trichoderma asperellum* isolates.

| Morphological and microscopic characters | *Trichoderma asperellum* isolates | | | |
|---|---|---|---|---|
| | Tas1 | Tas2 | Tas3 | Tas4 |
| Conidiation | ring like zones | ring to like zone | ring to like zone | ring to like zone |
| Conidiophores branching | branches typically paired | branches typically paired | branches typically paired | branches typically paired |
| Phialide shape | Flask-shaped | Flask-shaped | Flask-shaped | Flask-shaped |
| Phialide L/W ratio | 2.4 µm | 2.3 µm | 2.4 µm | 2.3 µm |
| Conidial shape | Subglobose to ellipsoidal | Subglobose to ellipsoidal | Subglobose to ellipsoidal | Subglobose to ellipsoidal |
| Conidia L/W ratio | 1.2µm | 1.1 µm | 1.2 µm | 1.1 µm |
| Conidial wall | smooth | smooth | Smooth | smooth |
| Appearance on agar | green to olive green | green | Green | dark green |
| Chlamydospore | found | found | found | found |
| Temperature optimum (PDA) | 28 °C | 28 °C | 28 °C | 28 °C |
| Colony radius on PDA at 280C after 72 h | 39 mm | 40mm | 41mm | 38mm |
| Pigmentation on medium | Not found | Not found | Not found | Not found |

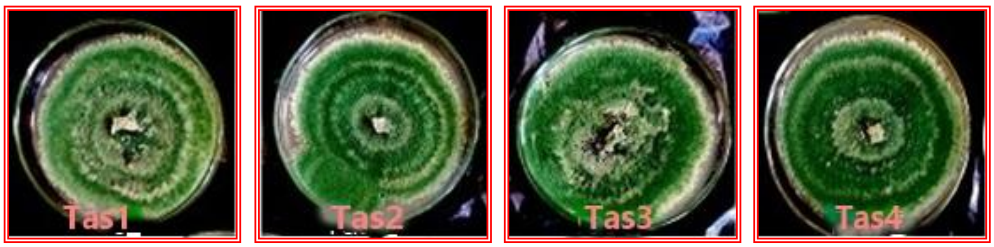



Fig. (1): Colony characteristics of *Trichoderma asperellum* isolates after cultured on potato dextrose agar (PDA) at 28°C for 7days.

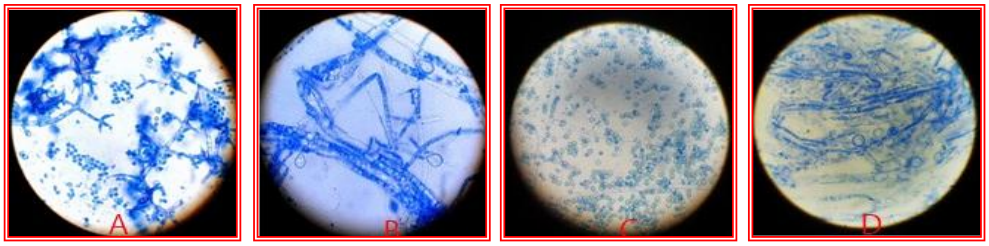



Fig.(2): Microscopic observation of conidiophore, conidia, spore and chlamydospores of *Trichoderma asperellum* isolates. (A) Conidiophore, (B) Conidia, (C) Spores, (D) Chlamydospores.


Fig. (3): Chitinase activity screening of *Trichoderma asperellum isolates* (Tas1, Tas2, Tas3 and Tas4) after seven days of inoculation in chitinase detection medium supplemented with colloidal chitin. C: control, B: The pictures were taken from the bottom side of petri dishes.

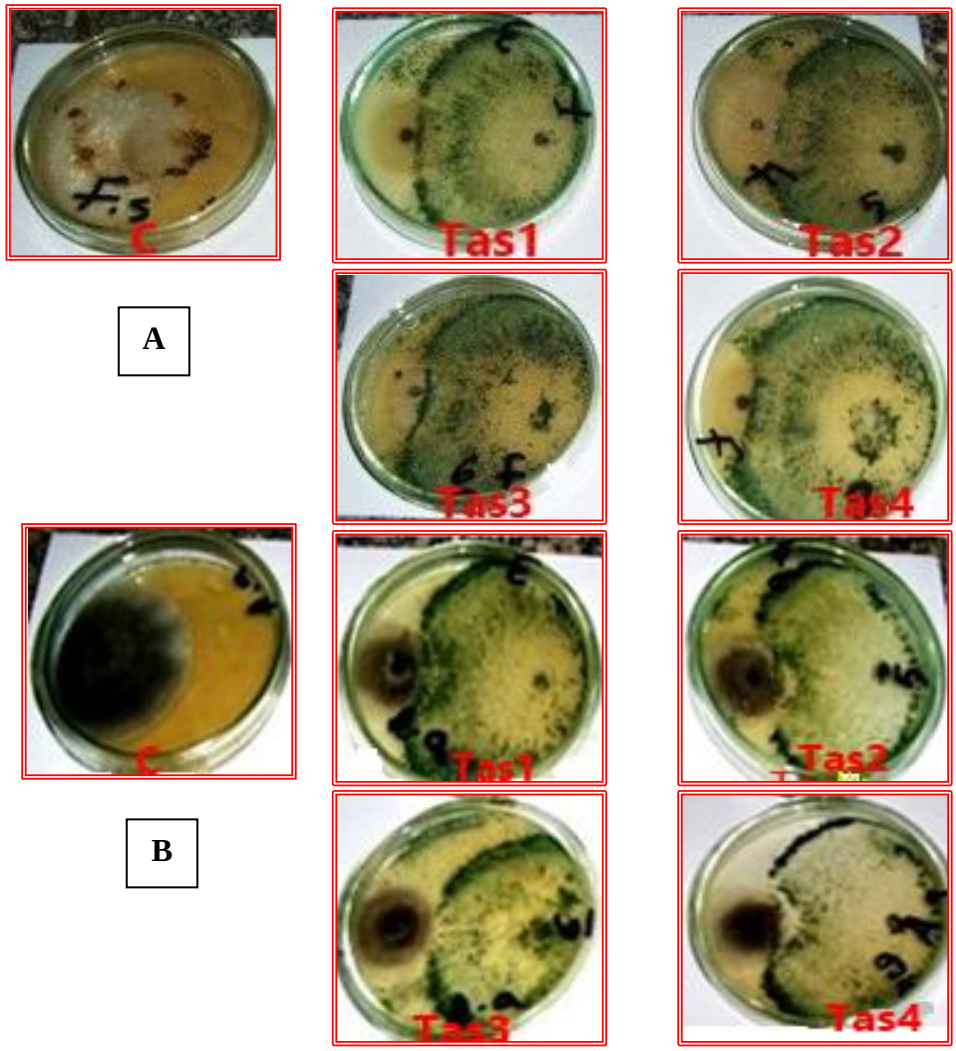
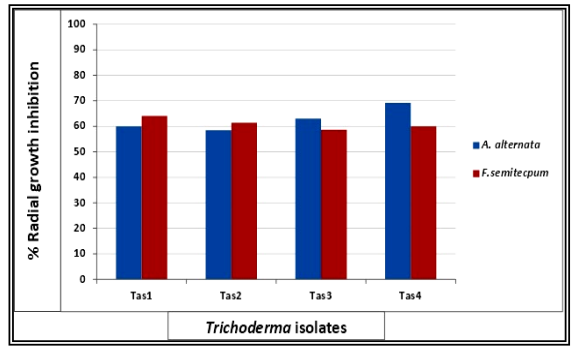



Fig. (4): Antagonistic activity of *Trichoderma asperillum* against *Fusarium semitectum* (A) and *Alternaria alternata* (B) evaluated interaction on dual culture.
C: Control [A: *F. semitectum* and B: *A. alternata* ], Tas1⟶Tas4: *T. asperillum* isolates.

Fig. (5): Antagonistic effect of *Trichoderma asperellum* strains on the percentage inhibition of radial colony growth of *Alternaria alternata* and *Fusarium semitectum* after seven days inoculation in dual culture.



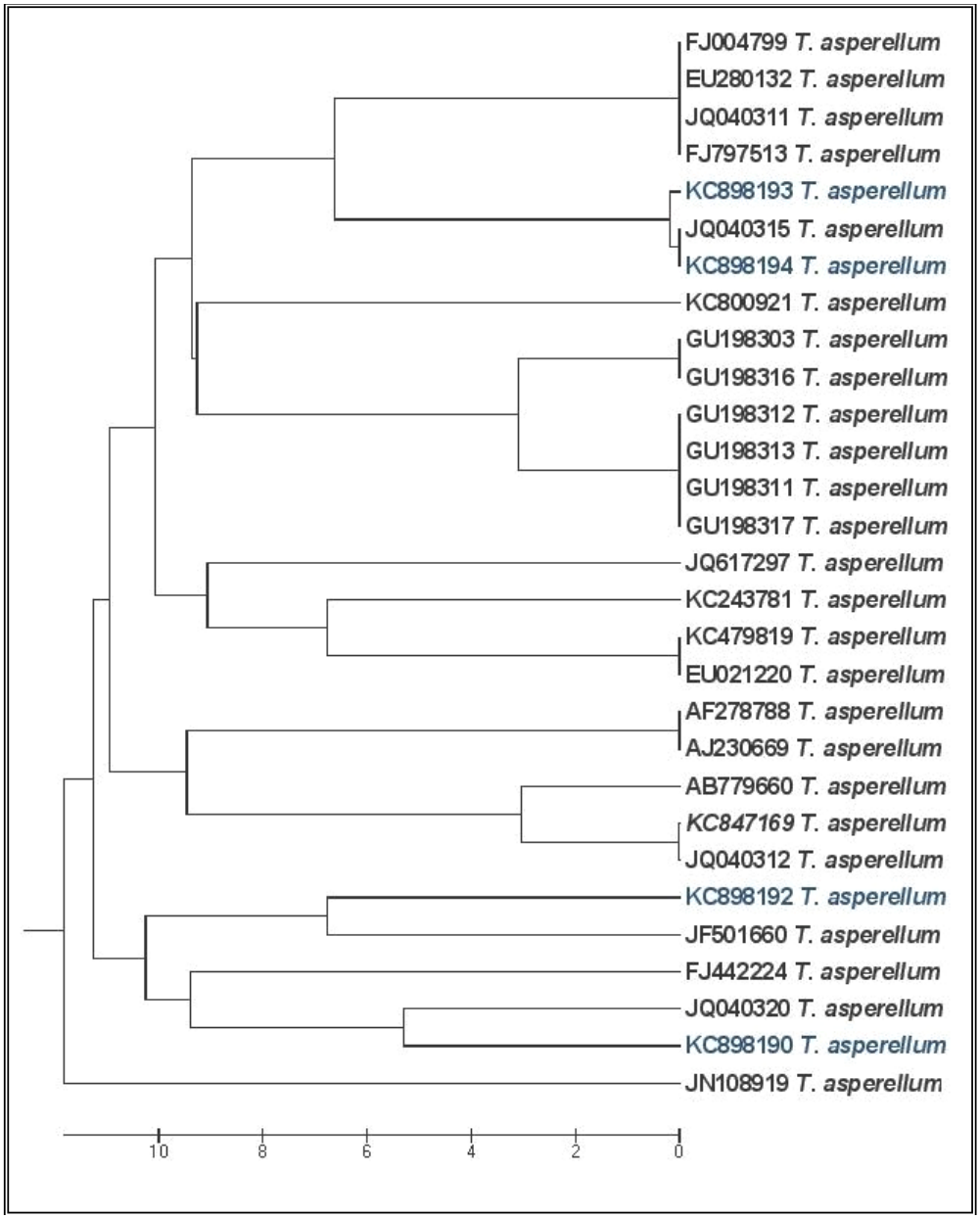



Fig. (6): The Phylogenetic tree obtained by sequence analysis of ITS1 and ITS4 sequence of the four *Trichoderma* isolates and the sequences of other 25 *Trichoderma* species obtained from GenBank