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ISOLATION AND MOLECULAR CHARACTERIZATION OF EGYPTIAN *TRICHODERMA* AND ASSESSMENT OF THEIR ANTAGONISTIC POTENTIAL AGAINST *RHIZOCTONIA SOLANI*

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ABSTRACT

Morphological and molecular characterization of antagonistic ability of *Trichoderma* species was studied. Soil dilution plate method was used to isolate *trichoderma* from rhizosphere of bean, cowpea, cucumber, wheat and faba bean plants. Based on morphological and cultural characteristics, the *Trichoderma* isolates were identified as *T. harzianum* (10 isolates), *T. koningii* (8 isolates), and *T. viride* (2 isolates). A portion of rDNA, 560-600 bp was amplified from six biocontrol isolates using ITS1 and ITS 4 primers, and was sequenced and aligned against ex-type strain sequences from TrichoBlast and established *Trichoderma* taxonomy. Molecular phylogenetic analysis were performed based on nucleotide sequences in order to examine these isolates among 15 accession numbers of *Trichoderma* spp. found in GenBank. The results indicate that the FUE3, FUE5, FUE6, FUE9 and FUE18 *Trichoderma* isolates are closely related to *Trichoderma koningii*, while FUE15 isolate is closely related to *Trichoderma harzianim* .This result was in accordance with the result obtained from morphological and cultural characteristics. Production of volatile inhibitors and mycoparasitism were investigated using *in vitro* and *in vivo* tests in dual culture PDA medium and infected soils. The percent inhibitory effect against growth of *Rhizoctonia solani* was calculated, *T. koningii* FUE3 showed the greatest antagonistic effect to the pathogen (57.77%) *in vitro* experiment whereas *T. koningii* FUE6 and FUE18 were gave the highest reduction 96% of disease incidence caused by *R. solani* in greenhouse conditions.

Keywords: *Trichoderma* species, *Rhizoctonia solani*,Ribosomal DNA, Internal transcribed spacer, Biocontrol

INTRODUCTION

The species of *Trichoderma* are well known and provide an effective biological control against several plant pathogens that cause major problems in the agricultural crops. The mechanisms of biocontrol including antibiosis, mycoparasitism and competition for nutrients have previously studied (**Ghisalberti and Rowland, 1993**; **Haran *et al.*, 1996**; **Simon and Sivasithamparam, 1989**). Species of the genus *Trichoderma* are attached to the host hyphae via coiling, hooks and aspersorium- like bodies, and penetrate the host cell wall by secreting several lytic enzymes (**kubicek *et al.*, 2001**). Mycoparasites produce cell wall degrading enzymes, which allow them to bore holes into other fungi and extract nutrients for their own growth. *Trichoderma* are a class of imperfect fungi, without known sexual stage. They are usually found in uncultivated land sometimes in forestland and to some extent in cultivated land. It is considered as one of the efficient biocontrol agent due to its high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in nutrient utilization, capacity to modify the rhizosphere, strong aggregativeness against the pathogenic fungi and efficiency in promoting plant growth and defense mechanisms **(Grondona *et al.*, 1997;Harman *et al.*, 2004)**. Morphological characterization of *Trichoderma* species is based on microscopic measurements of mycelia fragments as well as growth rates of different isolates on different media at different temperatures, while molecular identification is based on sequence comparisons of ITS regions of rDNA gene. The morphological characters of *Trichoderma* have been discussed by **Rifai (1969)** and **Bissett (1991).**They emphasizing the difficulties inherent in defining morphological species of *Trichoderma*. **Samuels (1996)** also provided detailed observations and comments on the utility of morphological characters to define species in *Trichoderma.* The morphological characteristics need to be combined with molecular data resulting from DNA sequencing **(Samuels, 2006).** The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) is one of the most reliable targets to identify a strain at the species level (**Kullnig-Gradinger *et al.*, 2002**). However, some closely related species share the sequences of their ITS regions, such as in *Trichoderma* sect. (**Samuels, 2006**). For identification of *Trichoderma* strains, *Trich*OKEY and *Trich*oBLAST (www.isth.info) are appropriate tools available online. Sequence data obtained from the ITS1 region of rDNA and a fragment of the translation elongation factor 1 (tef1) gene were used in a phylogenetic analysis. More than 50% of the potential biocontrol strains were grouped within *Trichoderma* sect. (**Hermosa *et al.*, 2004**).**Kindermann *et al.* (1998)** attempted a first phylogenetic analysis of the whole genus, using sequence analysis of the ITS1 region of the rDNA. However, the use of phylogenies based on single gene sequences is now generally discredited, especially as regards the use of ITS1 and/or ITS2, as some fungi and plants have been shown to contain analogous copies (**O'Donnell *et al.*, 1998; Lieckfeldt and Seifert, 2000**). Phylogeny of *Trichoderma* and phylogenetic relationships of its species were investigated by maximum parsimony analysis and distance analysis of DNA sequences from multiple genetic loci. 18S rDNA sequence analysis suggests that the genus *Trichoderma* evolved at the same time as *Hypomyces* and *Fusarium* and thus about 110 Myr ago. 28S rDNA sequence analysis shows that the genus *Trichoderma* is part of a monophyletic branch within the *Hypocreaceae* (**Gradinger *et al.*, 2002**).The molecular analysis of several strains revealed that the classification based on morphological data has been to a great extent, erroneous resulting in re-classification of several isolates and species, (**Kuhls, *et al.*,1996**).**Druzhinina and Kubicek (2005**) identified *Trichoderma* isolates according to the physiological, phenotypic characters and molecular markers. Therefore, this study aimed to: (1) Identify *Trichoderma* isolates isolated from rhizosphere in this study using morphological and molecular characters. (2) Evaluate the potential of isolates as biological control against Rhizoctoni *solani*.

MATERIAL AND METHODS

Isolation of *Trichoderma* isolates

Tweenty *Trichoderma* isolates were isolated from rhizosphere of bean, cowpea, cucumber, wheat and faba bean plants using a soil dilution plate method described by **Kucuk and Kivanc (2003).**

The morphological identification of *Trichoderma* isolates

Morphological identification of *Trichoderma* isolates was carried out according to an interactive key provided by **Samuels (2002)** at (http://nt.ars-grin.gov/taxadescriptions/keys/frameKey.cfm? gen=*Trichoderma*).*Trichoderma* isolates were identified to species level using the method described by **Harris (2000)** and confirmed by Assiut University Mycological Centre (AUMC), Assiut University,Egypt (www.aun.edu.eg/aumc/aumc.htm).

Antagonistic effect against *R. solani in vivo* and *in vitro*

Trichoderma isolates were evaluated for their potential to antagonize the plant pathogenic fungus *R. solani*. Bioassays were performed *in vitro* and *in vivo* according to the methods described by **Anees *et al*. (2010**) and **Ahmed *et al.* (2000)**.

Genomic DNA extraction from *Trichoderma* isolates

Genomic DNA was extracted from the mycelium of *Trichoderma* isolates using the method described by **Wijesinghe *et al.* (2010).**

PCR amplification of ITS region of *Trichoderma* isolates

To confirm the species of strain *Trichoderma* at the molecular level, ITS region was amplified using universal primers ITS 1(5'-TCTGTAGGTGAACCTGCGG-3')and ITS 4(5'-TCCTCCGCTTATTGATATGC-3'**)**according to**White *et al.* (1990)**and **Gardes and Bruns (1993).** Genomic DNA was amplified using a DNA thermal cycler of Applied BioSystems (USA).The reaction mixture contain 38.5 µl deionized water, 5µl 10 X Taq polymerase buffer, 0.5 µl of 1 U Taq polymerase enzyme, 3 µl 2 mM dNTPs, 1 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR conditions were as follows; an initial denaturation of 3 min at 94°C followed by 35 cycles of 1 min denaturation at 94 °C, 1 min primer annealing at 50 °C, 1 min extension at 72 °C and a final extension of 10 min at 72 °C. PCR products were checked by electrophoresis using 2% agarose gel in 1X TAE buffer. The PCR products were then purified by using Montage PCR Clean up kit (Millipore), following manufacture instructions.

Nucleotide sequencing and blast analysis

The purified PCR products of approximately 600 bp were sequenced by automated DNA sequencing reactions, which were performed using a sequencing ready reaction kit (Life Technology) in conjunction with ABI-PRISM and ABI-PRISM big dye terminator cycler. A consensus sequences were constructed by using the SeqMan^{TM} II (windows 32 Seq Man 4.05) package (DNA star). The sequence obtained in this study was submitted to the GenBank nucleotide sequence databases accession number: KC200070, KC200071, KC200073, KC200074 and KC200075 for *Trichoderma koningii* FUE3, *T. koningii* FUE5, *T. koningii* FUE6, *T. koningii* FUE9 and *T. harzianum* FUE15, respectively. Sequence identities were determined using both a specific database for *Trichoderma* and the Genbank general database. We successively used the different tools available online from the International Subcommission on *Trichoderma* and *Hypocrea* (ISTH, www.isth.info): TrichOKEY v. 2.0 based on an oligonucleotide barcode within the ITS1 and ITS2 sequences, TrichoMARK to analyse ITS, and TrichoBLAST to detect sequence similarity in the ITS region **(Druzhinina and Kubicek, 2005**). In some cases, blast analysis was also performed from the National Center for Biotechnology Information (NCBI) available online. Moreover, as per requirement, the alignments of sequences were performed with the help of the program CLUSTALW 2.1 multiple sequence alignment (**Larkin *et al*., 2007**) and sequences were manually edited by visual adjustments by the help of the computer program Seaview (**Galtier *et al.*, 1996**).

Phylogenetic analysis

These sequences were subjected toalignment with *Trichoderma* spp. sequences of the GenBank sequence database using the program BioEdit version 7.0.0 (**Hall, 1999**). The MEGA 4 program was used to generate a phylogenetic tree using the UPGMA method.

Statistical analysis

All data were statistically analyzed by one way analysis of variance (one way ANOVA) and post comparison was carried out with LSD test using SPSS (Statistical Package for Social Science) version 10. The results were expressed as mean ±SD.

RESULTS AND DISCUSSION

Isolation and Morphological identification of *Trichoderma* spp.

A total of 20 isolates of *Trichoderma* spp. were isolated from rhizosphere soil of different cultivation crops (Tab 1). Culture characteristics of *Trichoderma* isolates including comprising growth rate, colour and colony appearance were examined and summarized in (Tab 2). These characteristics were regarded as taxonomically characteristics for *Trichoderma suggested* by**Samuels *et al.* (2002**). Colony appearance of the three different species grownon PDA for 5 days at 28^{o}C was shown in (Fig 1). On PDA*T.harzianum*at the early stage whitish to greenish mycelia appeared. Next, a deep green colour developed in central part and gradually extended to the periphery, finally it appeared whitish green colour. Mostly globose conidia developed on phialides produced in the opposite direction in each point (Fig1A). On PDA *T. koningii* was formed whitish to pale green, hairy and flappy mycelial. Next pale green turned into whitish green to dark green colour. Branched conidiophores and dendroid conidiophores terminated by phialides carried confused ellipsoids to subglobose phialospores. Phialospores were pigmented, smooth, elliposide up to a little more than 4 mm long (Fig 1B). In *T. viride*, colony radius on PDA in darkness after 72h at 25°C 30-40 mm, after 144 h in darkness conidia form abundantly in conspicuous concentric rings. Phialides typically arising singly directly from the main axis or at the tip of a short lateral branch or in whorls of 2-3 at the tips of short branches, cylindrical to somewhat swollen in the middle and sometimes with an elongated neck, straight, hooked or sinuous (Fig 1C). The *Trichoderma* isolates could be classified into three groups based on culture and morphological characteristics descriptions by **Gams and Bissett (1998).** Representative isolates from each group were sent for identification by Assiut University Mycological Centre (AUMC), Assiut University, Egypt. These species of *Trichoderma* were identified as 10 isolates (*T. harzianum*), 8 Isolates (*T. koningii*) and 2 isolates (*T. viride*) (Tab 2). *T. harzianum* was the most frequently isolated species and it was recovered in nearly all the samples. In comparison to the growth of the *T. harzianum, T.koningii* and *T.viride* form mycelia on PDA, the conidia produced by *Tharzianum* and *T.viride* somewhat resembles each other. The length of phialides of *T. koningii* was longer than rest of the two species (Tab 2). All these description were in conformity as per given by **Gams and Bissett (1998).**

Table 1 Isolates of *Trichoderma* spp. used in the present study and their origin

Isolate code	Source of rhizosphere	Isolation date	Species
FUE11	Cucumber	June, 2004	*Trichoderma harzianum*
FUE2	Bean	July, 2004	*Trichoderma harzianum*
FUE7	Bean	July, 2004	*Trichoderma harzianum*
FUE14	Cowpea	July, 2004	*Trichoderma koningii*
FUE19	Cucumber	June, 2004	*Trichoderma viride*
FUE1	Faba bean	December, 2004	*Trichoderma harzianum*
FUE5	Faba bean	January, 2008	*Trichoderma koningii*
FUE6	Faba bean	January, 2008	*Trichoderma koningii*
FUE17	Faba bean	January, 2008	*Trichoderma harzianum*
FUE16	Faba bean	January, 2008	*Trichoderma koningii*
FUE4	Wheat	February, 2008	*Trichoderma harzianum*
FUE10	Wheat	February, 2008	*Trichoderma harzianum*
FUE3	Cucumber	May, 2008	*Trichoderma koningii*
FUE9	Cucumber	May, 2008	*Trichoderma koningii*
FUE15	Cucumber	May, 2008	*Trichoderma harzianum*
FUE8	Bean	August, 2008	*Trichoderma harzianum*
FUE13	Bean	August, 2008	*Trichoderma koningii*
FUE20	Cowpea	August, 2008	*Trichoderma viride*
FUE12	MERCEN*	-	*Trichoderma harzianum*
FUE 18	MERCEN*	-	*Trichoderma koningii*

Legend: *MERCEN,Faculty of Agriculture, Ain Shams University, Egypt.

Table 2 Morphological characteristics of *Trichoderma* spp. isolated from rhizosphere

Morphological characteristics	*Trichoderma harzianum*	*Trichoderma koningii*	*Trichoderma viride*
A-Conidia			
Shape	subglobose to ovoidal	oblong to narrowly ellipsoidal	Subglobose
Colour	Dark green	Green	Dark green
Length µm	2.7-3.5	3.7-4.5	3.5 -4.5
Width µm	2.4-2.9	2.8-3.2	3.2-3.8
Length/width ratio	1.1-1.2	1.3-1.4	1.0-1.2
B-Phialides			
Length µm	6.5-6.7	6.2-10.2	7.0-11.5
Midpoint µm	2.5-3.5	2.7-3.5	2.5-3.5

Efficacy of antagonistic against *R. solani in vitro*

The *Trichoderma* species were evaluated *in vitro* for their potential antagonizes the plant pathogenic fungus *R. solani*. The results of antagonism between *Trichoderma* spp. and *R. solani* are shown in (Tab 3). *Trichoderma* species were showed a significant reduction in mycelia growth of fungal colonies of *R. solani* face the *Trichoderma* spp. compared to the control. The results showed that the best antagonistic effect against the pathogen was obtained from *T. harzianum* and *T. koningii* isolates. The *Trichoderma koningii* FUE3 showed the highest inhibition (57.77%) of *R. solani* growth. On the other hand, *Trichoderma viride* FUE20 showed the lowest inhibition (25.33%). The antagonism was observed with the naked eye (Fig 2). One of the mechanisms of action *of Trichoderma* spp. which was observed to be adapted by *Trichoderma* to parasites *R. solan.i*

Figure 1 Colony appearance of three different species of *Trichoderma* grown for 5 days at 28 °C on PDA plate.
Legend: A-*T. harzianum*, B- *T. koningii*, C-*T. viride*

Table 3 Antagonistic effect of *Trichoderma* spp. isolates against growth of phytopathogen (*Rhizoctonia solani*) on PDA *in vitro*.

Isolate code	*Trichoderma* spp.	Pathogen edge (mm) (Mean ± S. E)	% inhibition
C (*R. solani*)		90.00 ^{A}	00.00
FUE3	*Trichoderma koningii*	31.67± 0.1202 ^{FG}	57.77
FUE15	*Trichoderma harzianum*	32.50± 0.1443 ^{FG}	56.67
FUE18	*Trichoderma koningii*	35.00± 0.0577 ^{EFG}	53.33
FUE5	*Trichoderma koningii*	35.00± 0.1155 ^{EFG}	53.33
FUE9	*Trichoderma koningii*	35.00± 0.2000 ^{EFG}	53.33
FUE6	*Trichoderma koningii*	35.33± 0.0882 ^{EF}	52.89
FUE14	*Trichoderma koningii*	35.50± 0.0289 ^{EF}	52.67
FUE4	*Trichoderma harzianum*	36.00± 0.0577 ^{DEF}	52.00
FUE17	*Trichoderma koningii*	36.33± 0.0882 ^{DE}	51.56
FUE1	*Trichoderma harzianum*	36.50± 0.1443 ^{DE}	51.33
FUE13	*Trichoderma koningii*	36.67± 0.0667 ^{DE}	51.11
FUE8	*Trichoderma harzianum*	37.00± 0.1155 ^{CDE}	50.67
FUE11	*Trichoderma harzianum*	37.33± 0.1202 ^{CDE}	50.23
FUE2	*Trichoderma harzianum*	38.33± 0.1764 ^{CDE}	48.89
FUE16	*Trichoderma harzianum*	38.50± 0.0289 ^{CDE}	48.67
FUE10	*Trichoderma harzianum*	38.67± 0.1333 ^{CDE}	48.44
FUE7	*Trichoderma harzianum*	39.50± 0.0289 ^{CD}	47.33
FUE12	*Trichoderma harzianum*	39.67± 0.0333 ^{CD}	47.11
FUE19	*Trichoderma viride*	40.67± 0.0882 ^{C}	45.77
FUE20	*Trichoderma viride*	56.00± 0.2000 ^{B}	25.33

Legend: * Means followed by the same letter (s) are not significantly different by LSD test. was by competition.

Trichoderma suppressed the growth of *R. solani* through the over growth. Insecond case, *Trichoderma* was observed to cluster around *R. solani* by the formation of small tufts thus limiting the growth of the pathogen of sheath blight. In both the cases formation of sclerotial bodies of *R. solani* were suppressed **(Shalini and Kotasthane, 2007**).The use of specific microorganisms that interfere with plant pathogens is a nature friendly, ecological approach to overcome problems caused by the chemical method of plant protection. Research has repeatedly demonstrated that phylogenetically diverse microorganisms can act as natural antagonists of various plant pathogens (**Cook, 2000**). According to our results, colonies of *T. harzianum* and *T. koningii* always grew faster than *R. solani* in single or mixed culture. Rapid growth of *Trichoderma* is an important advantage in competition with plant pathogenic fungi for space and nutrients (**Deacon and Berry, 1992**).

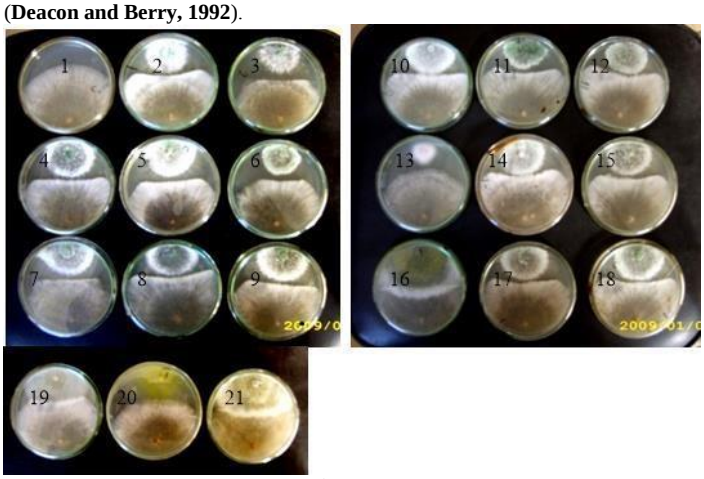

Figure 2 Antagonistic activity of *Trichoderma* species against *R. solani* evaluated interaction on dual culture.
Legend: 1- Control (*R. solani*) 2-*Trichoderma isolate FUE1 + R. solani* 3-*Trichoderma* isolate FUE2 + *R. solani*
4- *Trichoderma isolate FUE3 + R. solani* *5 -Trichoderma isolate FUE4 + R. solani*
6-Trichoderma isolate FUE5 + R. solani
7- Trichoderma isolate FUE6 + R. solani *8-Trichoderma isolate FUE7 + R. solani*
9-Trichoderma isolate FUE8 + R. solani
10- Trichoderma isolate FUE9 + R.solani *11-Trichoderma isolate FUE10 + R. solani*
12-Trichoderma isolate FUE11+ R. solani
13- Trichoderma isolate I12 + R. solani *14-Trichoderma isolate FUE13 + R. solani*
15-Trichoderma isolate FUE14 + R. solani
16- Trichoderma isolate FUE15 +R.solani *17-Trichoderma isolate FUE16 + R. solani*
18-Trichoderma isolate FUE17 + R. solani
19-Trichoderma isolate FUE18 + R.solani *20- Trichoderma isolate FUE19 + R. solani*
21- Trichoderma isolate FUE20 + R. solani

Efficacy of antagonistic against *Rhizoctonia solaniin vivo*

Selection of *Trichoderma* spp. for *in vivo* test was based on the *in vitro* results as explained above, such that FUE3, FUE15, FUE 18, FUE5, FUE9 and FUE6 were the best inhibition of *R. solani*. Results in (Tab 4) indicate that under greenhouse, treatments with *T. harzianum*, *T.koningii* significantly reduced the pre- and post-emergence damping off diseases incidence under artificial infection with *R. solani* in greenhouse conditions. The damping off disease incidence caused by *R. solani* under application of *Trichoderma* spp. were in the range of 4-16% and 58-74% compared to the control at pre- and post- emergence stages, respectively (Tab 4). The antagonistic effect of the six *Trichoderma* species against the faba bean root rot disease is shown in. (Fig 3). Results indicated that *Trichoderma* spp. significantly reduced the disease incidence at pre- and post- emergence stages in pot experiments. Treatments with *Trichoderma* spp. gave the highly protection of faba bean seedlings against damping off disease at post-emergences stage comparison with pre-emergence one. It is may be related to the ability of *Trichoderma* spp. to stimulate the enzymes in faba bean plants associated with increased the protection against disease. **Harman et al., (2004)** indicate that these fungi can induce systemic resistance in plants, thus increasing the plant defense response to diverse pathogen attack

Table 4 Effectof *Trichoderma* spp. treatment on the percentage of damping-off disease of faba bean plants under greenhouse conditions (artificial inoculation)

Trichoderma species		Disease assessment			
		Damping-off % pre-emergence (15 day)	Post-emergence (30 day)	Root rot (45 day)	Survival % (45 day)
Code	**Species**				
C*	Control	0^{C}	0^{C}	0^{B}	100^{A}
P	*R. solani*	36^{A}	83^{A}	100^{A}	0^{D}
FUE3	*T. koningii*	8^{BC}	74^{AB}	100^{A}	0^{D}
FUE5	*T. koningii*	12^{BC}	64^{B}	95^{A}	5^{C}
FUE6	*T. koningii*	4^{BC}	62^{B}	96^{A}	4^{C}
FUE9	*T .koningii*	12^{BC}	73^{AB}	100^{A}	0^{D}
FUE15	*T. harzianum*	16^{B}	71^{AB}	90^{A}	10^{B}
FUE18	*T .koningii*	4^{BC}	58^{B}	92^{A}	8^{B}

Legend: * plants grown in uninfected soil(without *R.solani* and/or *Trichoderma*)

Figure 3 Effect of *Trichoderma* isolates on the growth of bean plants at 25, 30 and 40 days after seeding.
Legend: c- Plants grown in uninfected soil (without *R. solani* and/or *Trichoderma*
p- Pathogen alone, 3-FUE3+ Pathogen, 5-FUE5+ Pathogen, 6-FUE6+ Pathogen, 9-FUE9+ Pathogen, 15- FUE15+ Pathogen, 18- FUE18+ Pathogen

PCR amplification of rDNA fragments

The rDNA fragments including 5.8S gene and the flanking intergenic transcribed spacer ITS region were amplified using ITS1 and ITS4 primers. A single fragment of approximately 600 bp nucleotide sequences was amplified from six isolates of *Trichoderma* (Fig 4).The results are in accordance with **Mukherjee *et al.* (2002**) who studied the identification and genetic variability of the *Trichoderm*a isolates. These results are also in accordance with several workers who observed the amplified rDNA fragment of approximately 500 to 600 bp by ITS-PCR in *Trichoderma* (**Ospina-Giraldo *et al.*, 1998; Venkateswarlu *et al.*, 2008**).

Identification of *Trichoderma* isolates

The morphological identification of *Trichoderma* isolates was complemented by a molecular identification based on internal transcripted spacers (ITS region) of rDNA sequences. The few morphological characters with limited variation may lead to an overlap and misidentification of the strains and showing the necessity of DNA based characters to complete identification evident from the present study.The ribosomal DNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (**Hibbett, 1992**). They also occur in multiple copies with up to 200 copies per haploid genome arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S and the 28S large subunit (LSU) genes. Internal, transcribed spacer (ITS) regions have been used to generate specific primers capable of closely related fungal species (**Bryan *et al.*, 1995**).The reliance on morphology or cultural characteristics for species determination and identification of fungi is difficult as the characteristics of isolation can change widely under varying environmental conditions (**Fernando *et al.*, 2009**). In fungi genomes, ribosomal DNA (rDNA) genes include the 18S, 5.8S and 28S segment that code for ribosomal RNAs (rRNA). These are highly conserved genes that are separated by two less conserved regions, the internal transcripted spacers 1 and 2 (ITS1 and ITS2). ITS1 sequences generally vary among different species, and used widely as informative regions for PCR assays. These ITS regions have several advantages for sequencing and phylogenetic analysis of fungal species. The rate of change is appropriate for studies at the species and genus levels, the alignment for the sequences is relatively simple and results can be interpreted phylogenetically. These regions are large enough to provide potential charactersits for phylogenetic reconstruction. Further, these ITS are flanked by regions that are highly conserved within genera and species **(Wijesinghe *et al.*, 2010**). Six isolates were identified at the species level by sequence analysis by ITS1 and 2 regions of the rDNA cluster. Sequence analysis of the ITS1 and 2 of rDNA has been especially reliable for the characterization of *Trichoderma* to the species level (**Samuels *et al.*, 2002**). There is an online method for the quick molecular identification of *Hypocrea/Trichoderma* at the genus, clade and species levels based on an oligonucleotide barcode: a diagnostic combination of several oligonucleotides (hallmarks) specifically allocated within the ITS1 and 2 sequences of rRNA repeat **(Druzhinina *et al.*, 2005**).Concerning molecular techniques, the GenBank database is generally referred, representing the largest reservoir of the sequences; however, it may not be safely used for identification as it contains many erroneous entries for *Trichoderma* (**Druzhinina and Kubicek , 2005**). This fact emphasizes the need of a specific database for *Trichoderma* containing only vouchered sequences, such as the ISTH (International Society on Thrombosis and Hemostasis) database has been used successfully for identification of *Trichoderma* strains (**Zhang *et al.*,2005 ; Migheli *et al.*, 2009**).

Table 5 Similarity coefficient percentage among six *Trichoderma* isolates.

isolates	FUE18	FUE9	FUE5	FUE6	FUE3	FUE15
FUE18	100					
FUE9	98.0	100				
FUE5	99.0	99.0	100			
FUE6	99.0	99.0	100.0	100		
FUE3	99.0	99.0	100.0	99.0	100	
FUE15	92.0	93.0	92.0	92.0	92.0	100

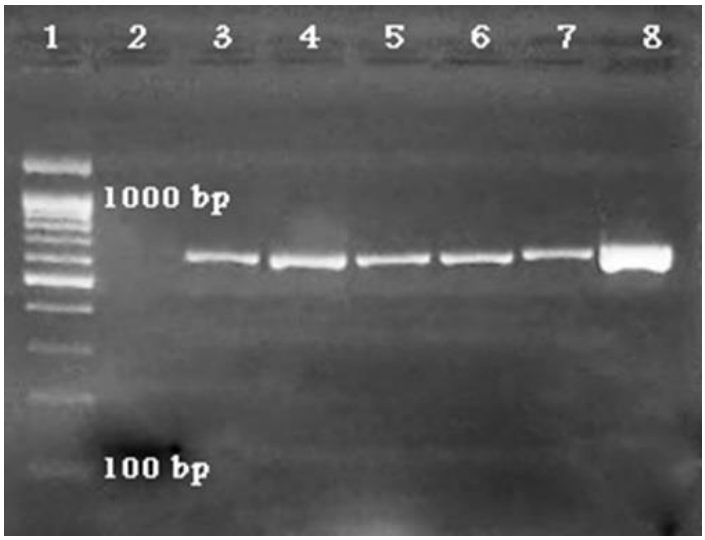

Figure 4 Agarose gel analysis of PCR products from amplification of ITS region of rDNA for *Trichoderma* straines**.**
Legend: Lane 1- 100bp DNA Ladder, Lane2- negative control, Lane3- FUE3, Lane4 - FUE5, Lane5-FUE6, Lane6- FUE9, Lane7- FUE15 and Lane8- FUE18

Nucleotide sequences and Blast analysis

Each sequence after editing was submitted to the Gene Bank and homology searches done against all the published *Trichoderma* sequences using BlastN and FASTA programs [National Center for Biotechnology Information (NCBI), USA]. Basic Local Alignment Search Tool (BLAST) search results of each sequence giving the closest match to the test sample was used to determine the species of *Trichoderma* isolates. Multiple sequence alignment was carried out including the ITS region of rDNA and gaps. There were quite a number of gaps introduced in the multiple sequence alignment within the ITS region of rDNA that were closely related, indicating a similar sequence (Fig 5). The highest homology (100%) was found between *Trichoderma* species (FUE5, FUE6 and FUE3, FUE5).While the less homology (92%) was found between isolate FUE15 and all isolates except isolate FUE9 (93%), (Tab 5). The sequences obtained in this study was submitted to the GenBank nucleotide sequence databases (Accession numbers: KC200070, KC200071, KC200073, KC200074 and KC200075) for *Trichoderma koningii* FUE3, *T. koningii* FUE5, *T. koningii* FUE6, *T. koningii* FUE9 and *T. harzianum* FUE15, respectively.

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FUE5-T.koningii      --------------------------------GCGGAGGGATCATTACCGAGTTTACAACT
FUE6-T.koningii      ----------------------------ACCAGCGGAGGGATCATTACCGAGTTTACAACT
FUE3-T.koningii      --------------------TTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACT
FUE9-T.koningii      --------------------TTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACT
I18-T.koningii       ----------AAGGTCTCCGTTGGTGAACGAGCGGAGGGATCATTCCCGAGTTTACAACT
FUE15-T.harzianum    AAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACT
                              ********************* ***************

FUE5-T.koningii      CCCCAAACCCAATG-TGAACGTTACCAAACTGTTGCCTCGGCGGGGTCAC-GCCCCGGGT
FUE6-T.koningii      CCCCAAACCCAATGGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCAC-GCCCCGGGT
FUE3-T.koningii      CCCCAAACCCAATG-TGAACGTTACCAAACTGTTGCCTCGGCGGGGTCAC-GCCCCGGGT
FUE9-T.koningii      CCCCAAACCCAATG-TGAACGTTACCAAACTGTTGCCTCGGCGGGGTCAC-GCCCCGGGT
I18-T.koningii       CCC-AAACCCAATG-TGAACGTTACCAAACTGTTGCCTCGGCGGGGTCAC-GCCCCGGGT
FUE15-T.harzianum    CCC-AAACCCAATG-TGAACGTTACCAAACTGTTGCCTCGGCGGGATCTCTGCCCCGGGT
             *** ********** ******************************** ** * *********

FUE5-T.koningii      GCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTA-GT
FUE6-T.koningii      GCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTA-GT
FUE3-T.koningii      GCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTA-GT
FUE9-T.koningii      GCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTA-GT
I18-T.koningii       GCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTA-GT
FUE15-T.harzianum    GCGTCGCAGCCCCGGACCAAGGCGCCCGCCGGAGGACCAACCAAAACTCTTATTGTATAC
             ***************** * ****************** * * * ********  ****

FUE5-T.koningii      CCCCTCGCGGACGTTATT-------------TCTTACAGCTCTGAGCAAAAATTC--AAA
FUE6-T.koningii      CCCCTCGCGGACGTTATT-------------TCTTACAGCTCTGAGCAAAAATTC--AAA
FUE3-T.koningii      CCCCTCGCGGACGTTATT-------------TCTTACAGCTCTGAGCAAAAATTC--AAA
FUE9-T.koningii      CCCCTCGCGGACGGTATT-------------TCTTACAGCTCTGAGCAAAAATTC--AAA
I18-T.koningii       CCCCTCGCGGACGTTATT-------------TCTTACAGCTCTGAGCAAAAATTC--AAA
FUE15-T.harzianum    CCCCTCGCGGGTTTTTTTATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAA
             **********   * **          ***   **    * *   **  ***

FUE5-T.koningii      ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
FUE6-T.koningii      ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
FUE3-T.koningii      ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
FUE9-T.koningii      ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
I18-T.koningii       ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
FUE15-T.harzianum    ATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
             ************************************************************

FUE5-T.koningii      AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT
FUE6-T.koningii      AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT
FUE3-T.koningii      AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT
FUE9-T.koningii      AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT
I18-T.koningii       AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT
FUE15-T.harzianum    AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT
             ************************************************************

FUE5-T.koningii      TGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCC
FUE6-T.koningii      TGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCC
FUE3-T.koningii      TGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCC
FUE9-T.koningii      TGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCC
I18-T.koningii       TGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCC
FUE15-T.harzianum    TGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCC
             ************************************************************

FUE5-T.koningii      CTCCGGGGGATCGGCGTTGGGGATCGGGA-CCCCTCACACGGGCGCC-GGCCCCTAAATA
FUE6-T.koningii      CTCCGGGGGATCGGCGTTGGGGATCGGGA-CCCCTCACACGGGCGCC-GGCCCCTAAATA
FUE3-T.koningii      CTCCGGGGGATCGGCGTTGGGGATCGGGA-CCCCTCACACGGGCGCC-GGCCCCTAAATA
FUE9-T.koningii      CTCCGGGGGATCGGCGTTGGGGATCGGGA-CCCCTCACACGGGTGCC-GGCCCCTAAATA
I18-T.koningii       CTCCGGGGGATCGGCGTTGGGGATCGGGAACCCCTCACACGGGCGCCCGGCCCCTAAATA
FUE15-T.harzianum    CTCCGGGGGGTCGGCGTTGGGGATCGGCCCTGCCTTG-GCGGTGGCC-GTCTCCGAAATA
             ********** ******************     ***    ***  *** * * ** *****

FUE5-T.koningii      CAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGC
FUE6-T.koningii      CAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGC
FUE3-T.koningii      CAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGC
FUE9-T.koningii      CAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGC
I18-T.koningii       CAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGC
FUE15-T.harzianum    CAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACA-CTCGCATCGGGAGC
             ********************************************** ******* ********

FUE5-T.koningii      GCGGCGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGTA
FUE6-T.koningii      GCGGCGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGTA
FUE3-T.koningii      GCGGCGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGTA
```

Figure 5 Nucleotide sequences alignment of the internal transcribed spacer (ITS region of rDNA) amplified from six *Trichoderma* species using CLUSTAL W 2.1 program

Phylogenetic analysis

Based on the results obtained all the six isolates can be grouped into two main clusters. One cluster represents *T. konigii* (FUE15) and other *T. harzianum* (FUE3, FUE5, FUE6, FUE9 and FUE18), (Fig 6).The topology of UPGAM tree of the six *Trichoderma* isolates with 15 accession numbers of *Trichoderma* in the GenBank database represented a monophyletic group (Fig 7). The five *Trichoderma koningii* (accession numbers: KC200070, KC200071, KC200073, KC200074 and KC200075) were clustered with ten accession numbers of *Trichoderma koningii*within this group, three isolates were closely related to four accession numbers of *Trichoderma harzianum* and formed a monophyletic lineage. *Trichoderma harzianum* (FUE15) was closely related to four accession numbers of *Trichoderma harzianum* and found a monophyletic lineage. The phylogenetic tree obtained by sequence analysis of ITS region of rDNA of six biocontrol *Trichoderma* strains and the sequences of fifteen other *Trichoderma* spp. obtained from sequence databanks is represented in (Fig 7). The ITS region of rDNA sequence was chosen for this analysis because it has been showed to be more informative with various sections of the genus *Trichoderma* (**Ospina-Giraldo *et al.*, 1998**).There are 21 *Trichoderma* isolates could be divided into four groups. Group 1, the *T.koningii- Hypocera koningii*, This group contains two subgroups one of them include *Trichoderma koningii*-Z79628, *Trichoderma koningii*-X93983, *Hypocrea koningii*-AJ301990, *Hypocrea koningii*-AY154931 and *Hypocrea koningii*-EU280128. The other subgroup includes the *Hypocrea koningii*-HQ607942, *Hypocrea koningii*-HQ608000, *Hypocrea koningii*-HQ608031 and *Trichoderma koningii*-AF456923 isolates. Group 2 includes two strains; include one of our biocontrol strains *Trichoderma koningii* FUE9 and Hypocrea *koningii*-AF538622. Group 3 was contained 4 of our biocontrol strains *Trichoderma koningii* FUE18 in the first sub subcluster. On the other hand, the second sub subclusters include *Trichoderma koningii* FUE5, *Trichoderma koningii* FUE6 and *Trichoderma koningii* FUE3. Group 4 includes the biocontrol strains *Trichoderma harzianum* FUE15, which are grouped with the representative strain *Trichoderma harzianum*AF194011; *Trichoderma harzianum*AF194009; *Trichoderma harzianum* AF194008; *Trichoderma harzianum*AF443922 in the first second sub sub group, the first sub sub group include *Trichoderma harzianum* AY154949 strain. The ITS PCR has helped to detect polymorphism at ITS region of rDNA among the *Trichoderma* isolates. Sequencing of ITS-1 and ITS-2 of the rDNA gene complex was undertaken because these regions are known to be highly variable (**White *et al.*,1990; Bruns *et al.*, 1991**) and suitable for phylogenetic studies of fungi at the inter- and intraspecific level **(O'Donnell, 1992; Egger and Sigler, 1993**).

CONCLUSION

The interactive morphological key and a specific molecular database coupled with tools for identification of *Trichoderma* strains represent an ideal way to identify the *Trichoderma* spp., although, they need regular updates to include the rapidly increasing number of species of this genus.

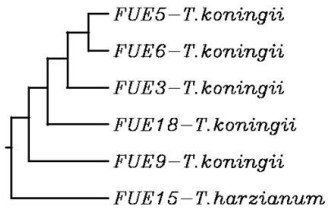

Figure 6 The Phylogenetic tree showing the relationship between the six *Trichoderma* strains. The rooted phylogenetic tree was constructed using the CLUSTAL W 2.1 multiple sequence alignment programs. Rooted phylogentic tree (UPGMA)

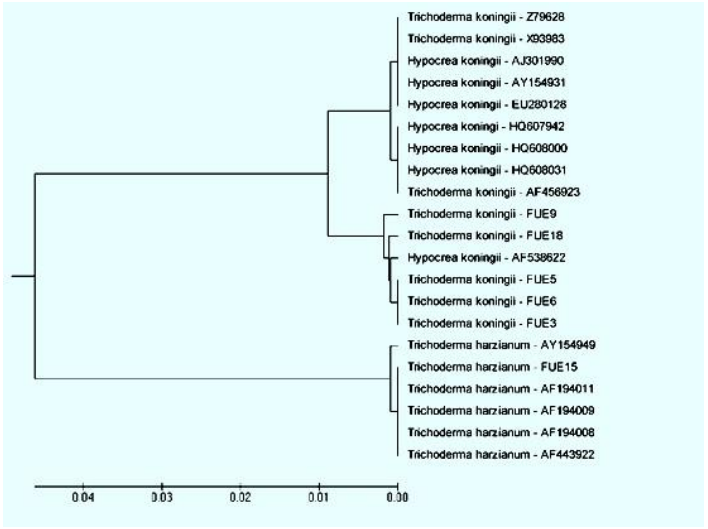

Figure 7 Phylogenetic tree showing the relationship between *Trichoderma* isolates. The tree was constructed using MEGA4 sequence alignment programs. Rooted phylogentic tree (UPGMA).

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