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GENETIC DIVERSITY OF *TRICHODERMA* ISOLATES AND THEIR ANTAGONISM AGAINST *RHIZOCTONIA SOLANI* AND *PYTHIUM APHNIDERMATUM*

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ABSTRACT: *The PCR-based Random Amplified Polymorphic DNA (RAPD) analysis was used to evaluate the genetic relatedness among 12 isolates of Trichoderma obtained from soil rhizosphere of different geographic locations in Egypt. The similarity coefficient values among the isolates ranged from 0.25 to 0.93. The Antagonistic ability of Trichoderma isolates against Rhizoctonia solani and Pythium aphnidermatum as soil borne plant pathogens was studied in dual culture plates. The results showed that the isolates were more active in inhibition growth of P. aphnidermatum than R. solani. Trichoderma TZ2 was the most efficient isolate in inhibition growth of P. aphnidermatum and R. solani of 78% and 73% respectively. The Trichoderma isolates TK and TM3 showed the lowest inhibition activity against P. aphnidermatum and R. solani respectively.*

***Key words**: RAPD-PCR, biological control, Pathogenic Fungi*

INTRODUCTION

Biological control proved to be the most effective treatment to control soil borne pathogenic fungi than the excessive application of pesticides which cause environmental pollution and several health problems. *Trichoderma* is one of excellent biological control agents that exhibit effective antagonism against wide range of soilborne plant-pathogenic fungi (Gupta *et al.*, 2010; Kubicek and Harman 1998; Barari, 2016). *Trichoderma* can inhibit pathogen growth in soil rhizosphere through reducing pathogen infection via different mechanisms like mycoparasitism, competition and antibiosis (Singh 2006; Mukhopadhyay 2009). For example, Sadykova *et al.* (2015) tested the antibiotic activity of 42 strains that represent 8 species of the *Trichoderma* (*T. asperellum, T. viride, T. hamatum,T. koningii, T. atroviride, T. harzianum, T. Citrinoviride*, and *T. longibrachiatum*) isolated from Siberian. They showed that the strain *T. citrinoviride* TV41, exhibited high antibiosis activity against the pathogenic fungi as *Candida albicans*genus and *Aspergillus*. *Rhizoctonia solani* and *Pythium aphnidermatum* are among the dangerous pathogenic fungi that cause diseases such as seed rot and seedling damping-off for a variety of crops (Kamala and Indira 2011). Siameto *et al.* (2011) evaluated the antagonism of sixteen isolates of *T. harzianum* against different pathogenic fungi (*R. solani*, *Pythium sp.*, *Fusarium graminearum, F. oxysporum f. sp Phaseoli* and *F. oxysporum f. sp Lycopersici*). They found that nine of the sixteen isolates were able to inhibit the growth of three pathogens each by more than 50 percent. In addition, Bazgir and Okhovat (1996) used *T. virens, T. harzianum* and *T. viride* to control of *R. solani* on *Phaseolus vulgaris* beans and they noted that *Trichoderma* can reduce the level of disease when added to soil one month before sowing. Furthermore, *T. harzianum, T. koningii* showed high antagonistic abilities in inhibition fruit rots pathogens of sapodilla (*Manilkara zapota L.*) *(*Bhale *et al.*, 2013). Several studies used molecular markers tools to cluster and prove taxonomy based-morphology data (Shahid *et al.*, 2014). RAPD is useful marker which has been used to study *Trichoderma* fingerprints (Nagee *et*

al., 2003; Salama *et al.,* 2002; Gupta *et al*., 2010 and Chakraborty *et al.,* 2011). Moreover, RAPD marker was used to distinguish the isolates of *Trichoderma* and results were found to be consistent with each the morphological and physiological data (Fujimori and Okuda, 1993; Zimand *et al.,*1994). The present study is aimed to characterize the twelve isolates of *Trichoderma* obtained from different geographic locations of Egypt using PCR-based RAPD and also to evaluate their antagonistic abilities against *R. solani* and *P. aphnidermatum* as soil borne plant pathogens.

MATERIALS AND METHODS

Isolation and morphological identification of *Trichoderma*

Twelve cultures of *Trichoderma* were isolated from rhizosphere of cultivated soils from different Egyptian governorates as shown in Table (1) according to the dilution plate's method (Elad *et al*., 1981). These isolates were first morphologically identified based on conidiophore branching pattern and conidium morphology as described by Rifai (1969), Barnett and Hunter (1998) and Bissett (1991a,b,c).

Soil borne pathogens

The pathogenic fungi isolates of *Rhizoctonia solani* and *Pythium aphnidermatum* were kindly provided by Plant Pathology Department, Faculty of Agriculture, Menoufia University, Shibin El-Kom, Egypt.

DNA extraction from *Trichoderma* isolates

DNA isolation of *Trichoderma* was performed according to the procedures of Al-Samarrai and Schmid (2000). The quantity and quality of DNA was tested by estimation the A260/A280 ratio and checked on agarose gel.

Table 1. The *Trichoderma* isolates and their isolation sources.

Trichoderma isolates	Code	Source of isolation
T. koningii	TK	Ismailia governorate
*T. hamatum*1	TM1	Menoufia governorate
*T. hamatum*2	TM2	Menoufia governorate
*T. hamatum*3	TM3	Gharbia governorate
*T. hamatum*4	TM4	Sharkya governorate
T. viride 1	TV1	Gharbia governorate
T. viride 2	TV2	Kafer El-shikh governorate
T. viride 3	TV3	Sharkya governorate
T. viride 4	TV4	Menoufia governorate
T. harzianum 1	TZ1	Sharkya governorate
T. harzianum 2	TZ2	Ismailia governorate
T. harizianum 3	TZ3	Menoufia governorate

PCR Reaction and Amplification Conditions

For RAPD analysis, ten random primers were used as indicated in Table 2 (supplied by Sigma, Egypt). Reactions of PCR were carried out in 25 µl volume containing 1 µl of diluted genomic DNA (50 ng), 2.5 µl of Taq 10X buffer (10 mM_Tris-HCl pH 8.0, 50 mM KCl and 2 mM $MgCl_2$), 1 µl of 10 µM of deoxynucleotides triphosphate mix (dNTPs), 4 µl of primer (10 pmol), 1 µl of *Taq* DNA polymerase (5 U $µl^{-1}$) and sterile deionized distilled water (up to a total volume of 25 µl). For DNA amplification, the thermal cycler (Progene) was programmed at 94°C for 4 min; 35 cycles Each cycle consisted of 1 min at 94°C, 1 min at 34°C and 2 min at 72°C, followed by a final extension time of 7 min at 72°C. Amplified DNA products were analyzed by electrophoresis in 1.5% agarose gel run in TBE for 90 m at 120V using 100 Base plus DNA marker Ladder. DNA was stained with ethidium bromide (05 µg ml^{-1}) and photographed under UV light.

Antagonism of *Trichoderma* isolates

The antagonism of *Trichoderma* isolates against *R. solani* and *P. aphnidermatum* was evaluated by testing three criteria in dual culture plate technique (Coskuntuna and Ozer 2008). One week-old culture of *Trichoderma* isolates and pathogens was used as a source of inoculums. Mycelial discs (5mm-diameter) of *Trichoderma* isolates, *R. solani* and *P. aphnidermatum* were placed on opposite sides (15 mm from the edges) of PDA plates (80 mm). The cultures were incubated at 28 °C until the growth of the pathogen covered completely the control plates. First, the radial growth inhibition of pathogen(s) was taken as index of antagonistic ability and calculated using the following equation:

$$(R1 - R2)/ R1 \times 100$$

Since R1 is maximum radial growth of the pathogen, while R2 is the radial growth of the pathogen opposite the isolates of *Trichoderma* (Zhou and Reeleder, 1990). Second, over growth ability was calculated as mycelial growth of *Trichoderma* over the pathogenic ones and measured starting from the contact zone by milimeter (pe`er and Chet 1990). Third, inhibition zones were measured by observing formation of clear zones between *Trichoderma* isolates and pathogenic ones.

Table 2. The list of primer sequences used for RAPD.

Primer	Sequence
OPA-02	5'- TGCCGAGCTG-3'
OPB-07	5'- GGTGACGCAG-3'
OPB-08	5'- GTCCACACGG-3'
OPB-09	5'-TGGGGGACTC-3'
OPB-18	5'-CCACAGCAGT-3'
OPB-19	5'-ACCCCCGAAG-3'
OPG-04	5'-AGCGTGTCTG-3'
OPG-07	5'-GAACCTGCCC3-3'
OPE-04	5'- GTGACATGCC-3'
OPF-06	5'-GGGAATTCCC-3'

Data and cluster analysis

The cluster analysis was done by NTSYS-pc version 2.11 W program (Rohlf 1998) based on Jaccard's similarity coefficient (Jaccard, 1908). Dendrogram was constructed according to the Unweighted Pair-Group Method with Arithmetical average (UPGMA). The obtained data of radial growth inhibition and over growth were statistically analyzed byANOVA using SPSS Statistical Package with Duncan's multiple-range test at 5% level of Significance.

RESULTS AND DISCUSSION

Morphological and molecular characterization of *Trichoderma* isolates

Twelve isolates of *Trichoderma* were isolated from soil rhizosphere of agricultural fields represents different locations in Egypt (Table 1). These isolates were first morphologically identified according to conidiophore branching pattern and conidium morphology and were found to be represent four species of *Trichoerma; T. viride, T. hamatum, T. harzianum* and *T. Koningii* (Table 1). The genetic diversity among the twelve isolates was tested by PCR based RAPD analysis using 10 random primers as indicated in Table 2. The amplified products gave a total of 70 reproducible bands ranging from approximately 500 to 3000 bp and about 96 % of these bands were polymorphic (Fig 1, Table 4). The similarity coefficient values ranged from 0.25 to 0.93. The highest similarity was observed between isolates of *T. hamatum* (TM2) and *T. harzianum* (TZ2) with similarity coefficient value of 0.93, while the lowest value was observed between the isolates *T. hamatum* (TM4) and *T. harzianum* (TZ3) with similarity coefficient value of 0.25 (Table 3). The diversity between the isolates agrees with observations of Moller *et al.* (1995) who found intra specific diversity not only among isolates of *Chaunopycnis alba* collected from different geographic locations, but also among isolates from the same location. The generated dendogram showed that the twelve isolates can be grouped into two main clusters (Fig 2). The first cluster represents most of the tested isolates (TK, TM1, TM2, TM3, TV1, TV2, TV3, TV4, TZ1, TZ2 and TZ3), The second cluster contained only the isolate of TM4. These results are consistent with previous studies employed RAPD markers to estimate the genetic variation among *Trichoderma* isolates and found them genetically similar (Gupta *et al*., 2010; Gopal *et al*., 2008).

Evaluation of *Trichoderma* antagonism activity

The antagonistic activity of all tested isolates against two common pathogenic fungi *R. solani* and *P. aphnidermatum* was recorded by testing three criteria, radial growth inhibition, over growth and inhibition zone in dual culture plate technique (Coskuntuna and Ozer 2008). *P. aphnidermatum* is one of the common causal pathogen of damping-off disease of beans (*Phaseolus vulgaris L*), while *R. solani* affects the cotton seedlings (Kamala and Indira 2011) and the two pathogens are destructive soilborne pathogens of many crops worldwide. The obtained results showed that these isolates are more active against *P. aphnidermatum* than *R. solani* (Table 5 and Fig 3*)*. In general, the isolate TZ2 was the most efficient isolate in growth inhibition of each *P. aphnidermatum* and *R. solani* with inhibition percentages of 78% and 73% respectively. In contrast, the isolate TK gave the lowest inhibition activity against *P. aphnidermatum*, while the isolate TM3 showed the lowest inhibition activity against *R. solani*.

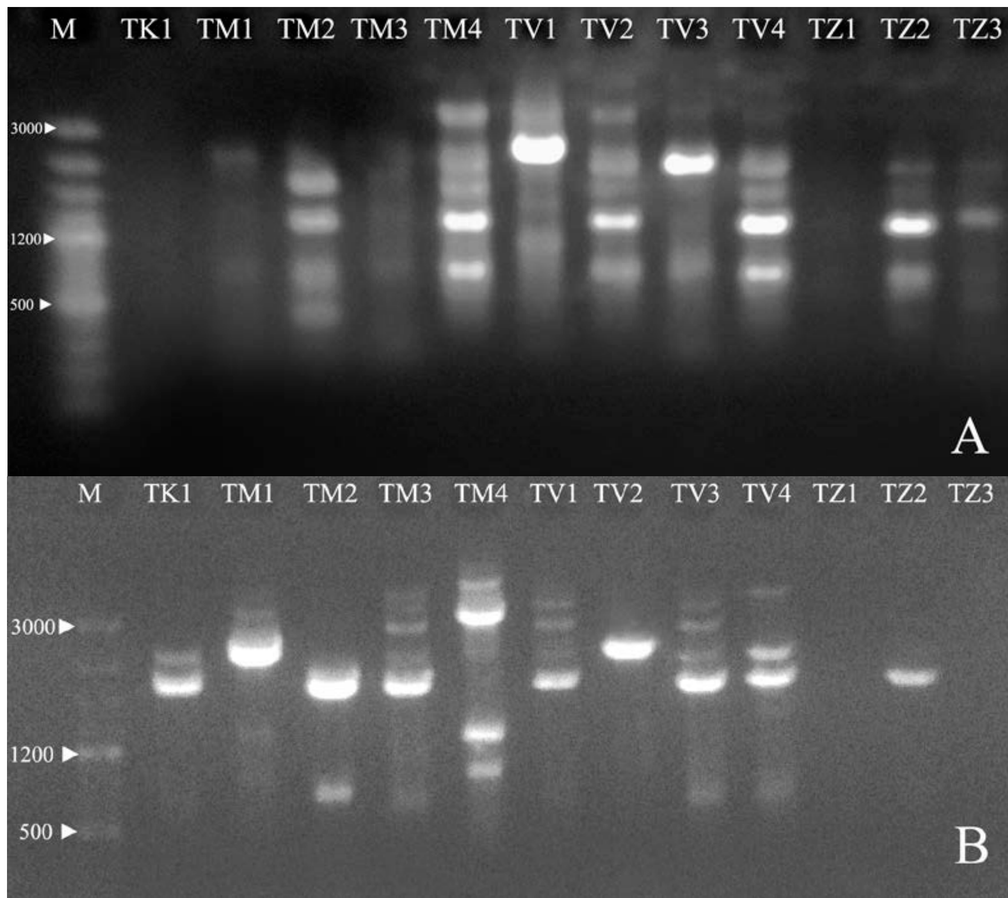

Figure 1. RAPD profiles of twelve *Trichoderma* isolates, M (100 Base plus DNA marker Ladder); OPB-09 Primer (A) and OPE-04 primer (B).

Table 3. The similarity matrix among the twelve isolates of *Trichoderma.*

Isolates	TK1	TM1	TM2	TM3	TM4	TV1	TV2	TV3	TV4	TZ1	TZ2	TZ3
TK	1.00											
TM1	0.78	1.00										
TM2	0.84	0.62	1.00									
TM3	0.75	0.71	0.59	1.00								
TM4	0.28	0.37	0.31	0.40	1.00							
TV1	0.68	0.71	0. 53	0.68	0.28	1.00						
TV2	0.68	0.65	0.71	0.56	0.40	0.56	1.00					
TV3	0.84	0.81	0.81	0.78	0.25	0.71	0.65	1.00				
TV4	0.75	0.71	0.84	0. 75	0.34	0.62	0. 68	0.90	1.00			
TZ1	0.81	0.78	0.65	0.81	0.28	0.62	0.75	0.78	0.68	1.00		
TZ2	0.90	0. 68	0.93	0.65	0.31	0.59	0.71	0.81	0. 78	0.71	1.00	
TZ3	0.84	0.81	0.75	0.71	0.25	0. 65	0.71	0.81	0.78	0.84	0.81	1.00

Table 4. RAPD analysis of *Trichoderma* isolates

Primer	Size of bands (bp)	Total number of bands	Number of polymorphic bands	Percentage of polymorphic bands (%)
OPA-02	500-2000	6	6	100
OPB-07	500-2000	7	7	100
OPB-08	500-3000	5	3	60
OPB-09	500-3000	8	8	100
OPB-18	700-3000	7	7	100
OPB-19	500-2000	7	7	100
OPG-04	500-3000	8	8	100
OPG-07	500-2000	7	7	100
OPE-04	800-3000	8	8	100
OPF-06	500-2000	7	6	86
Total		70	67	96

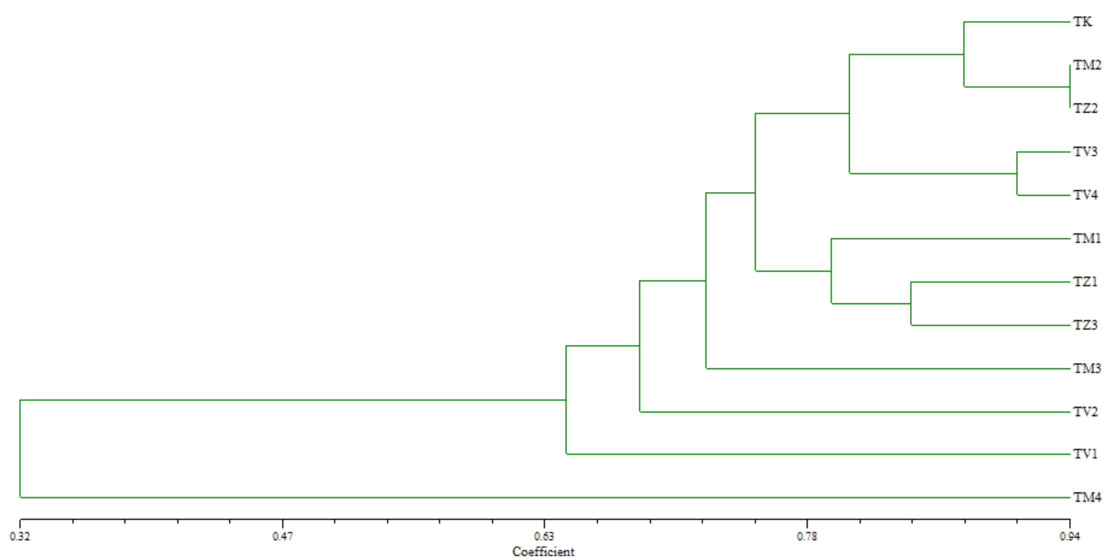

Figure 2. Dendrogram showing the genetic relationships among twelve *Trichoderma* isolates based on RAPD analysis.

Table 5. Evaluation of radial growth inhibition, over growth and inhibition zones formation for *Trichoderma* isolates against *R. solani* and *P. aphnidermatum* in dual culture plate technique (Coskuntuna and Ozer 2008).

Isolates	Radial growth inhibition(%)		Over growth (mm)		Inhibition zone	
	P. aphnidermatum	*R. solani*	*P. aphnidermatum*	*R. solani*	*P. aphnidermatum*	*R. solani*
TK	$64.4^{d} \pm 1.1$	$60.7^{e} \pm 1.5$	$17.7^{bcd} \pm 1.5$	N	N	+
TM1	$71.4^{b} \pm 0.0$	$67.6^{abc} \pm 0.6$	$17.3^{cd} \pm 0.6$	N	N	+
TM2	$67.4^{c} \pm 1$	$64.7^{d} \pm 0.6$	$18^{bc} \pm 1$	N	N	+
TM3	$66.9^{c} \pm 1.3$	$55^{f} \pm 1$	$18^{bc} \pm 1.5$	N	N	+
TM4	$70.4^{b} \pm 1\ .7$	$70.4^{ab} \pm 1.7$	$20^{a} \pm 1$	N	N	+++
TV1	$71.2^{b} \pm 0.2$	$68.1^{abc} \pm 0.2$	$18^{bc} \pm 0.6$	N	N	++
TV2	$71.3^{b} \pm 0.2$	$69.3^{ab} \pm 0.6$	$18^{bc} \pm 0$	N	N	+++
TV3	$66.6^{c} \pm 0.7$	$66.^{3c} \pm 0.6$	$17^{cd} \pm 1.7$	N	N	+
TV4	$72.1^{b} \pm 1.8$	$69^{ab} \pm 1$	$18^{bc} \pm 0$	N	N	++
TZ1	$72.2^{b} \pm 1.8$	$67.2^{bc} \pm 0.8$	$19.3^{ab} \pm .2$	N	N	++
TZ2	$78^{a} \pm 1.5$	$73^{a} \pm 1$	$16^{d} \pm 2$	N	N	+++
TZ3	$72.3^{b} \pm 1.6$	$67.7^{abc} \pm 1.2$	$17.3^{cd} \pm 0.6$	N	N	++

+ + +, wide inhibition zone; + +, moderate inhibition zone; +, narrow inhibition zone; N, non-inhibition zone.
* Means within classification followed by different letters are significantly different at 0.05 level.

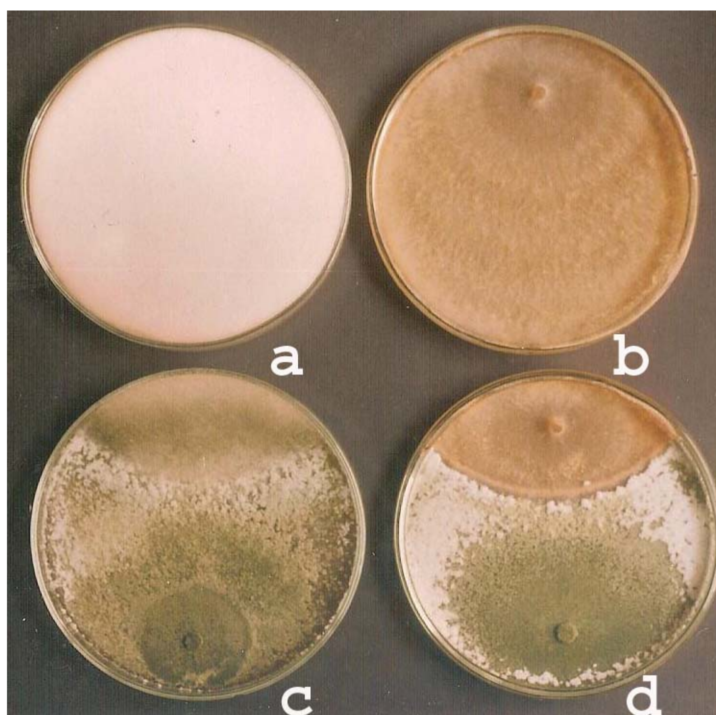

Figure 3 . The antagonistic abilities of *Trichoderma* against *R. solani* and *P. aphnidermatum* pathogens; *P. aphnidermatum* control (a); *R. solani* control (b); *Trichoderma* isolate inhibit growth of *P. aphnidermatum* (c) and *Trichoderma* isolate inhibit growth of *R. solani* (d).

For the results of over growth, the isolates TZ1 and TM4 exhibited the highest abilities for over growth on *P. aphnidermatum,* while the other isolates varied in their over growth abilities and all the tested isolates failed to grow on *R. solani.* Finally, For inhibition zone formation, it must be mentioned that no inhibition zones were found between all of the tested isolates and *P. aphnidermatum*, although wide and moderate inhibition zones were formed in the case of *R. solani.* Interestingly, the results showed that the isolates TZ2, TV2 and TM4 that were active in inhibition of pathogens growth formed wide inhibition zones. *Trichoderma spp.* use different mechanisms to attack the other fungi including mycoparasitism, competition for nutrients, and production of different toxins and antibiotics (Daniel and Filho, 2007; Elad *et al.*, 1999; Haran *et al.*, 1996; lorito and scala 1999; Sivasithamparam and Ghisalberti, 1998). Moreover, the hydrolytic enzymes secreted by some *Trichoderma spp.* such as chitinases and cellulases play an important role in destruction of pathogens cell wall and thereby inhibition their growth (Benítez *et al.*, 2004 and Brunner *et al.*, 2003; Thrane *et al.*, 1997). Since the cell wall of *Pythium* species is composed of cellulose (Bartinicki-Garcia 1968), while chitin is the main structural component of *Rhizoctonia solani* cell walls (Farkas 1990; Sivan and Chet 1989), This can explain why the tested isolates are more active against one pathogen than another. It can be said that the composition of pathogen cell walls and each quality and quantity of *Trichoderma* secreted enzymes determine the aggressiveness of *Trichoderma* against pathogens. On the other side, the presence of inhibition zones between *R. solani* and *T. isolates* is consequence of secretion of diffusible non-volatile inhibitory substance by the *T. isolates (*Siameto *et al.,* 2011*).* The previous studies revealed that the antimicrobial metabolites produced by *Trichoderma* are effective against a wide range of fungal phytopathogens as *F. oxysporum*, *R. Solani* and *P. aphnidermatum* (Xiao-Yan *et al.*, 2006; Zivkovic *et al.*, 2010).

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التباين الوراثى لعزلات الترايكودرما وتضادها الحيوى ضد الريزوكتونيا سولانى والبيثيم افندرماتم

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الملخص

استخدمت تقنية الرابد المعتمدة على تفاعل البلمرة المتسلسل لتقييم العلاقة الوراثية بين 12 عزلة من فطر الترايكودرما والتى تم الحصول عليها من تربة تمثل عدة اماكن جغرافية مختلفة فى مصر. وقد تراوحت قيم التشابة بين العزلات من 0.25 الى0.93 . كذلك تم دراسة قدرة التضاد الحيوى لهذة العزلات ضد الفطريات الممرضه الريزوكتونيا سولانى والبيثيم افندرماتم وقد اوضحت النتائج ان هذة العزلات اكثر فاعلية فى تثبيط الفطر الممرض البيثيم افندرماتم عن الفطر الريزوكتونيا سولانى. العزله TZ2 كانت الافضل فى تثبيط فطر البيثيم افندرماتم بنسبة 78% وكذلك تثبيط الفطر الريزوكتونيا سولانى بنسبة 73%. وعلى الجانب الاخر العزلات TKوTM3 كانتا الاضعف فى تثبيط كلا الفطريين الممرضيين

الكلمات المفتاح: الرابد، المقاومة الحيوية ، الفطريات الممرضه