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CHARACTERIZATION OF *Trichoderma* spp. BY RAPD AND BIOCONTROL ABILITY TO ROOT ROT PATHOGENS OF WATERMELON

Awad, M.A.[1]; S.M. El-Desouky[2]; Majdah M.Y. Al-Tuwaijri[3] and S.M. El-Ganainy [2]

1- Agric. Bot. Dept., Fac.of Agric., Minufiya Univ, Shibin El-Kom, Egypt.

2- Plant Pathology Res. Inst., Agric. Res. Center, 12619 Giza, Egypt

3- Fac. of Applied Sciences for Girls, Umm Al-Qura Univ., Makkah, KSA.

ABSTRACT

Eleven isolates belonging to genus Trichoderma were isolated from the rhizosphere of watermelon roots grown at different localities in Egyptian soil. They were morphologically identified as *T. harzianum, T. hamatum, T. glaucum, T. reesei and T. viride.* These five species comprised different eleven isolates which were originated from different localities. significant differences in radial growth of the different pathogens tested. Also, there was a significant difference between the pathogens in dual culture and the control treatment which include the pathogen only. Results lead us to conclude that DNA based RAPD provide a reliable method for grouping isolates of *Trichoderma* spp*.* In addition, analyzing phyllogenetic similarities among isolates based on DNA pattern according to RAPD technique. Genomic DNA prepared from the eleven *Trichoderma* spp*.* isolates was used to screen primer for readily detectable and reproducible polymophic PCR amplifications. The PCR conditions employed, allowed amplification of many bands on the agarose gel. Bands present in each sample were scored for presence and absence of amplification products. UPGMA cluster analysis divided in two subclusters; the first one included the *T. harazianum* isolates. However, the second cluster included the *T. hamatum* and *T. glaucum* isolates. Meantime, each isolate of *T. reesei* and *T. viride* fall in one another subcluster showing the lowest similarity with other isolates. Results conclude that the differences in DNA profiles among isolates of *Trichoderma* species not related to their geographic origin or biocontrol ability. The biocontrol ability of the tested isolates of *Trichoderma* species under greenhouse condition, against the different pathogens using the susceptible watermelon cultivar Giza 1 were studied. Results obtained showed the effectiveness of all isolates of *Trichoderma* species in this respect. Results also showed the superiority of *T. harzianum* and *T. hamatum* isolates as well as *T. reesei* in some cases against the tested pathogens.

Keywords: *Trichoderma* spp., root rot, watermelon, RAPD.

INTRODUCTION

Biological control offers a save alternative approach to the chemical control of phytopathogens. biocontrol means reduction in survival or activity of the pathogen or a pest resulting from interaction(s) with living organisms it plays an important role in pathogen inoculum reduction by attacking pathogen structures. Mechanism of biocontrol activity has most often involves one or more natural processes e.g. antibiosis, parasitism, competition or lyses. Thus, biocontrol agents have become one of the most important components of plant disease control. Also, under field conditions, many soil borne fungi affect the activity of other fungi. Such fungi influence the natural balance of

the soil borne microorganisms. Therefore, such fungi of antagonistic characters have been used in the biocontrol of the soil borne pathogens. One of the most important biocontrol agents in the soil borne fungi are *Trichoderma* spp. which were isolated from plant rhizosphere as soil borne fungi. There are numerous reports on the use of *Trichoderma* spp. in controlling root rot pathogens (Xue *et al*. 1995; Miller and Bruton, 1998; El-Desouky *et al*.2000; Patricio *et al*. 2001; Ji *et al*. 2002; Howell, 2003 and Xue, 2003). Use of random amplified polymorphic DNA (RAPD based-PCR) to asses the genetic diversity and phylogenetic relationships between different *Trichoderma* spp. isolates as well as to distinguish the variation in antagonistic ability of the different *Trichoderma* spp. isolates were reviewed by many investigators (Sivakumar *et al.* 2000; Salama *et. al.*2002 and Maymon *et al.* 2004)

Therefore, the present investigation aims to study the antagonistic activity of *Trichoderma* species isolates in dual culture against different root rot pathogens of watermelon. Random amplified polymorphic DNA (RAPD based PCR) studies were carried out on DNA patterns of different *Trichoderma* spp. isolates using random amplified polymorphic DNA (PCR-based RAPD). Also, antagonistic activity against some pathogens in pots under greenhouse condition was carried out.

MATERIALS AND METHODS

Isolation of the root rot fungi:

Diseased roots of watermelon plants showing root rot symptoms were collected from different growing locations in Egypt. Isolation was done using tissues excised from cortical lesions of tap root and lateral roots. The surface sterilized samples were plated on potato dextrose agar (PDA) medium and incubated at 25˚C for 3-5 days. The growing fungal colonies were transferred to PDA. Pathogenic fungi were identified using the morphological and microscopical characteristics according to Barnett (1960); Domsch *et al*. (1980) and Nelson *et al*., (1983).

Isolation of *Trichoderma* species:

The Dilution Plate Method (DPM) was used for the isolation of *Trichoderma* spp. Soil and rhizosphere samples were taken from watermelon fields by uprooting the infected plants with great care to obtain most of the intact root system. The root system was shaken gently to get rid of most of the adhering soil particles. Trichoderma Selective Medium (TSM) was used for isolation of *Trichoderma* species from soil (Elad *et al.,* 1981). The developed colonies were marked and transferred onto PDA slants. *Trichoderma* spp. were identified after growing them on 20% malt extract agar which they were incubated for two days at 25°C according to Rifai (1969) and Bissett (1991).

Random amplified polymorphic DNA analysis of *Trichoderma* spp. isolates:

This experiment was carried out in the Fingerprinting laboratory, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt.

DNA extraction from *Trichoderma* spp. isolates: DNA isolation of *Trichoderma* spp. was performed according to the procedures of Al-Samarrai and Schmid (2000). For fungal growth synthetic complete medium Anjani and Panda (1995). Then 50 ml of medium in 100 ml flask was inoculated with each single spore isolate and incubated for 3 days at 28 ºC with shaking. Then mycelium was harvested by vacuum and washed with distilled water and immersed in liquid nitrogen. 250 mg of fresh weight mycelium were ground to a fine powder in liquid nitrogen using a pre-cooled pestle. The ground mycelium was transferred to an Eppendorf tube and re-suspended and lysed in 500 µl of lysis buffer by pipetting with a Gilson P 1000 pipetman (set to 1000µl) until the viscosity of the suspension was significantly reduced. Then the formation of froth indicated the detachment of DNA from polysaccharides. RNase A (2 µl of 10 mg/ml) was added and the mixture was incubated for 5 min. at 37 ºC. To facilitate the precipitation of most polysaccharides, protein and cell debris, 165 µl of 5 M NaCl solution was added and the components mixed by inverting the tubes several times. The suspension was centrifuged at 14000 rpm for 20 min at 4 ºC. The supernatant was immediately transferred to a fresh tube and 400µl chloroform and 400 µl phenol were added. The solution was mixed by gently inverting the tube until the solution becomes milky (usually >50 times). After centrifugation for 20 min., the aqueous phase was transferred to a fresh tube and extracted with an equal volume of chloroform. The DNA in the aqueous supernatant was precipitated with two volumes of 95% ethanol. To free the DNA from polysaccharide, the precipitate was re-suspended in 500 µl of lysis buffer and mixed by gentle pipetting. Then 165 µl of 5 M NaCl solution were added and the tube gently inverted several times. The suspension was then chloroform-extracted as described above. Usually, after centrifugation for l0 min, the aqueous phase was clear and the DNA was precipitated with 95% ethanol. On rare occasions, the aqueous phase was still cloudy, in which case it was re-extracted with on volume of chloroform before the DNA precipitation. The precipitated DNA washed three times with 70% ice-cold ethanol, dried and dissolved in 50 µl deionized water and stored at -20 ºC.

RAPD procedure: RAPD technique based on the polymerase chain reaction (PCR) was used to detect RAPD markers using arbitrary 10 - mer primer according to Williams *et al*. (1990). The primer was selected randomly from a group of sequences with a 60% to 70% (G+C) content and no self-complementary ends. Because this primer is 10 nucleotides long, it has the possibility of annealing at a number of locations in the genome. Sequence of this primer was as follows: 5'GTTTCGCTCC3'

Preparation of PCR reactions: Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech) were used for PCR reactions. 10 ng of genomic DNA template, 20 microliter of the primer and sterile distilled water were added to a total volume 25 µl to the bead. Reactions were carried out according to the

methods of Williams *et al*. (1990), with some modifications. Amplification was carried out in a DNA themocycler (PCR unit II Biometra).

PCR program and temperature profile: The thermal cycler programmed as follows: 1- An initial strand separation cycle at 95 °C for 5 minutes. 2- The next 45 cycles were composed of a denaturation step at 95°C for 1 min, an annealing step at 36°C for 1 min and polymerization step at 72 °C for 2 min. 3- The final cycle was a polymerization cycle performed at 72 °C for 5 min.

Amplification product analysis: The PCR products analyzed by electrophoretic separation in 1% agarose gel in 1X TAE buffer. The samples were loaded into the wells of the gel and marker DNA (PCR Promega G317A) was loaded on the side of the gel to determine the sizes of the DNA pattern. The PCR size marker produces fragments with precisely known molecular weights namely; 1000, 750, 500, 300, 150 and 50 base pairs. Gel was run at 75 volts for 3-4 hours and was stained with ethidium bromide. Finally, the RAPD patterns were visualized with an UV- transilluminator and photographed by a Polaroid camera.

Differences among patterns were scanned for band R_f using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6C, Fullerton CA 92631). The mobility of each fragment was measured and recorded and the size in bp of each fragment produced was estimated.

DNA Data analysis: Data analysis was conducted using the Numerical Taxonomy and Multivariate Analysis system, Version 2.1 (NTSYSpc) according to Rolhf, (1993). Cluster analysis was then conducted on the genetic similarities matrix with un-weighted pair-group method based on arithmetic average (UPGMA) to develop a dendrogram.

Antagonistic ability of *Trichoderma* species in dual culture:

The antagonistic ability of 11 isolates of *Trichoderma* spp. was tested against seven root rot pathogens of watermelon in dual culture. Cultures were incubated in the dark at 25ºC until the growth of the pathogen covered completely the check plates. The inhibition of pathogen(s) growth was taken as an index of antagonistic ability, which was calculated by comparing radial growth of the pathogen colony directly to the biocontrol fungi colony with maximum radial growth according to Zhou and Reeleder, (1990);

Inhibition % = $[(R_1 – R_2) / R_1] \times 100$ where,

R_1 = is the maximum radius of the pathogen colony.

R_2= is the radius of the pathogen colony directly opposite the biocontrol agent colony.

Antagonistic ability of *Trichoderma* spp. against some root rot pathogens in pots under greenhouse conditions:

Different isolates of each of five *Trichoderma* species were selected to study their effect(s) as biocontrol agents against some watermelon root rot pathogens i. e. *F. solani* and *R. solani* under greenhouse conditions. Preparation of inocula of the different isolates of the pathogens and *Trichoderma* spp. was done as mentioned before. Two weeks before sowing,inocula of *Trichoderma* spp. isolates were individually mixed thoroughly with non-sterilized field sandy loamy soil at the rate of 3% of soil weight (w/w).Soil was infested with the individual isolates of the pathogens,

one week before sowing.The infested soil was watered and covered with plastic sheets in the shade under greenhouse conditions.Soil infested with the individual isolates of *Trichoderma* spp. isolates and pathogens isolates as well as their combinations was placed in plastic pots (25 cm diameter). Check treatment was infested soil only with individual isolates of pathogens. Seven watermelon seeds of the cv. Giza 1 were sown in each pot and each treatment was replicated 4 times. Disease incidence was recorded as root rot severity (Aegerter *et al*., 2000) and disease severity index (Kobriger *et al*., (1998) as well as number of surviving plants were estimated at the end of the growing period.

RESULTS

Trichoderma spp. isolates obtained were identified as *T. harzianum*, *T. hamatum*, *T. glaucum*, *T. reesei* and *T. viride*. These five species comprised different eleven isolates which were originated from different localities as follows:

1- *T. harzianum* was the most frequent specie which represented by six isolates obtained from different localities i.e. isolates TZ1 and 2 from Queisna (Minufiya); isolate TZ3 from Sadat (Minufiya); isolate TZ4 from El-Kasassin (Ismailiya); isolates TZ5 and 6 from El-Kanater (Qalubiya).
2- *T. hamatum* was represented by two isolates i. e. isolate TM1 selected from the isolates obtained from Minufiya governorate (Queisna province). While isolate TM2 selected from isolates obtained from El-Kanater province (Qalubiya).
3- *T. glaucum* was represented by one isolate (TG1) obtained from Nubariya (Beheira).
4- *T. reesei* was represented by one isolate (TR1) obtained from El-Kasassin (Ismailiya).
5- *T. viride* was represented by one isolate (TV1) obtained from Queisna (Minufiya).

Antagonistic activity of *Trichoderma* species in dual culture:

The effect of different isolates of five *Trichoderma* spp. i.e. *T. harzianum, T. hamatum*, *T. glaucum*, *T. reesei* and *T. viride* on radial growth and the percentage of inhibition toward the different watermelon root rot pathogens was studied in dual culture in Petri dishes. Different isolates of each *Trichoderma* spp. were tested individually against seven root rot pathogens i. e. *F. solani*, *R. solani*, *F. oxysporum* f. sp. *nevium*, *F. moniliforme*, *M. phaseolina*, *M. cannonballus* and *P. aphanidermatum* (Table, 1).

Results given in Table (1) showed significant differences in radial growth of the different pathogens tested. Also, there was a significant difference between the pathogens in dual culture and the control treatment which include the pathogen only.

T1

All *T. harzianum* isolates showed percentage of radial growth inhibition reached less than 50% with different pathogens. *T. harzianum* isolate (TZ4) showed the lowest percentage of radial growth inhibition with *F. oxysporum* f. sp. *nevium* (27.76%). On the contrary TV4 and TV5 showed the highest percentage of inhibition (57.64 and 52.22%, respectively) with the pathogen *F. moniliforme*. All isolates of *T. harzianum* showed moderate effect on the percentage of radial growth inhibition with other pathogens.

On the other hand, *T. reesei* and *T. viride* isolates showed the highest percentage of inhibition reached more than 50% in many cases. and TR1 showed 61.14% and TV1 showed 64.63 respectively with *M. cannonballus* as well as they showed 60.27% and 56.06 with *F. moniliforme* and 50.17 and 50.90% with *P. aphanidermatum*. Meanwhile, they were less than 50% with the other four pathogens i. e. *F. solani*; *R. solani*; *F. oxysporum* f. sp. *nevium* and *M. phaseolina*.

Two isolates of *T. hamatum* showed moderate effect on the inhibition of the pathogens growth which showed the high effect on *F. moniliforme* where TM1 and TM2 showed 54.30 and 56.05%, respectively. But the percentage of inhibition was lower with the other pathogens; it reached 35.40 and 40.89% on *F. solani*; 43.28 and 37.88% on *M. cannonballus*; 40.02 and 38.78 on *P. aphanidermatum*. Moderate effect was obtained on *R. solani* (47.62 and 48.01%); on *F. oxysporum* f. sp. *nevium* (46.92 and 49.21%); on *M. phaseolina* (47.63 and 47.13). On isolate of *T. glaucum* (TG1) showed moderate effect on the inhibition of the pathogens growth. It showed inhibition percent started from 28.64% on *F. solani* and 35.26% on *M. phaseolina*. But it reached more than 40% on other different pathogens.

Random amplified polymorphic DNA (RAPD) analysis of *Trichoderma* spp. isolates:

In the present work, we used random amplified polymorphic DNA analysis obtained by electrophoretic techniques. Also, we used a computerized program for cluster analysis to differentiate among DNA profiles of eleven *Trichoderma* species isolates obtained from the rhizospher of watermelon roots grown in different localities in Egypt. These banding patterns of *Trichoderma* species isolates RAPD are shown in (Fig. 1).

Genomic DNA prepared from the eleven isolates of *Trichoderma* species was used to screen primer for readily detectable and reproducible polymorphic PCR amplifications. The PCR conditions employed in this study allowed amplification of many bands on the agarose gel. Bands present in each isolate were scored for presence and absence of amplification products. The primer was chosen to screen the eleven *Trichoderma* spp. isolates. This primer is widely used in the RAPD experiments which had proven to detect polymorphism in the absence of specific nucleotide in *Trichoderma* species isolates (Fig. 1). To illustrate the genetic distance among *Trichoderma* species isolates, a phylogenetic tree was obtained using UPGMA clustering method. The dendrogram (Fig. 2) indicated that the clustering of *Trichoderma* species isolates were consistent with the data obtained from the morphological identification of *Trichoderma* species isolates.

The dendrogram constructed based on similarity levels (SLs) generated from cluster analysis of RAPD showed in Fig. (2). The dendrogram

arranged isolates of *T. harzianum* which they belonged to remotely related one subcluster. Also isolates of *T. hamatum* and *T. glaucum* belonged to relate another subcluster, while the isolates of *T. viride* and *T. reesei* were not related to them and showed low similarity to the two subclusters mentioned before.

The dendrogram was divided into two subcluster (SL = 53.40). The higher the SL, the more closely isolates of *T. harzianum* which were isolated from Queisna and Sadat (Minufiya) from El-Kasassin (Ismailiya) and El-Kanater (Qalubiya) which formed a single subcluster, while the isolates of *T. hamatum* and *T. galacum* formed the another subcluster. The isolates of *T. viride* and *T. reesei* showed to be not related to them. But TR1 showed low similarity to the two subclusters mentioned before (SL = 39.83%), while, isolate TV1 showed very low similarity to other all isolates (SL = 34.56%).

The observed variation among isolates show that grouping of *Trichoderma* species isolates in these subclusters was not related to their geographic origin of isolates.

Isolates of *T. hamatum* (TM1 and TM2) showed higher SL, the more closely isolates (SL = 94.65%) although they were originated from different localities. Isolate TM1 from Minufiya governorate (Queisna province) and TM2 from El-Kanater (Qalubiya province). *T. glaucum* (TG1) obtained from Nubariya (Beheira) were related to the two isolates of *T. hamatum* showing SL = 63.25% which all they were related to the six isolates of *T. harzianum* showing SL = 53.40%.

Also, the observed variation in DNA profiles among isolates was not related to their biocontrol ability. For example, isolates of *T. harzianum* nos., TZ 1, 2, 3, 4, 5 and TZ 6 showed different levels of biocontrol ability showing different values of inhibition % with different root rot pathogens; however, they belonged to remotely related subclusters. Another example was the belonging of isolate of *T. viride* TV1 obtained from Queisna (Minufiya) was not related to other subclusters, although it showed almost the same biocontrol ability with TR 1. Also the isolate of *T. reesei*; TR 1 obtained from El-Kasassin (Ismailiya) which occupied one subcluster and were not related to other subclusters, although they showed almost the same biocontrol ability with TV 1. Results led us to conclude that the differences in DNA profiles among isolates of *Trichoderma* species were not related to their geographic origin or biocontrol ability.

Antagonistic ability of *Trichoderma* spp. against root rot pathogens in pots under greenhouse conditions:

In this experiment, four species representing eleven isolates of the biocontrol agents *Trichoderma* spp. i. e. *T. harazianum*, *T. hamatum*, *T. glaucum*, *T. reesei* and *T. viride* were tested as soil treatment for their effects against the most virulent isolates of seven root rot pathogens of watermelon i.e. *F. solani* (isolate No. 3) and *R. solani* (isolate No. 2) as shown in (Tables 2 and 3). They were tested as biocontrol agents against root rot incidence of the susceptible watermelon cv. Giza 1 in pot experiments under greenhouse condition. Soil used in this experiment was a non-sterilized field sandy loamy soil infested with each pathogen separately. The check treatment used was naturally soil inoculated with each pathogen in a separate experiment.

Generally, all species of *Trichoderma* significantly affected the incidence of the disease in terms of the number of healthy and infected plants. Also, disease index values were assessed.

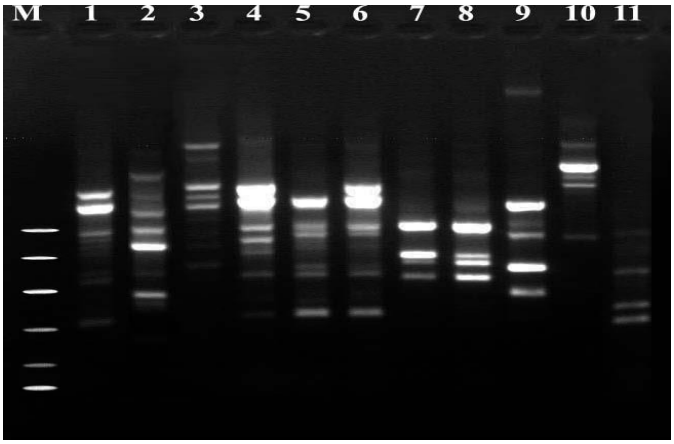

Fig. (1): Polymorphic DNA bands for eleven *Trichoderma* species isolates amplified with random primer and electrophoresed in 1% agarose gel.
M = DNA marker; 1 to 6 = isolates of *T. harzianum*; 7&8 = two isolates of *T. hamatuum*; 9 = one isolate of *T. glaucum*; 10 = one isolate of *T. reesei*; 11 = one isolate of *T. viride*.

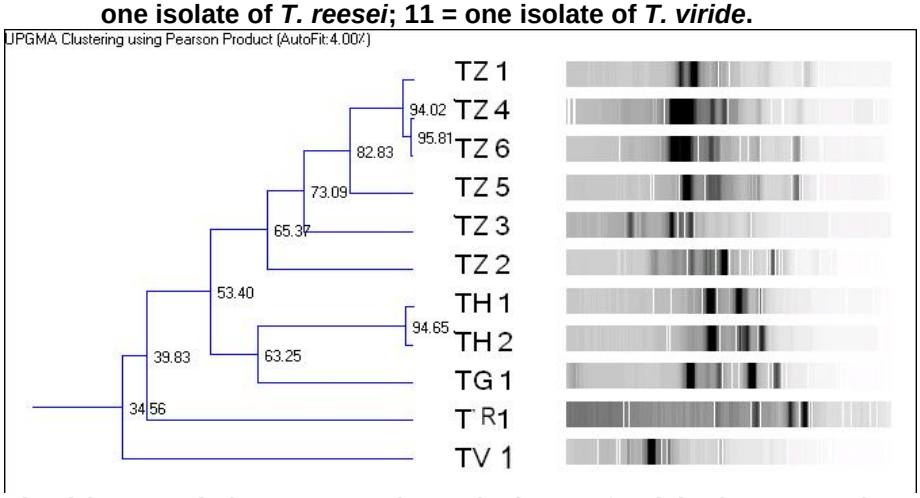

Fig. (2): Association among eleven isolates of *Trichoderma* species isolated from rhizosphere of watermelon roots grown in different localities in Egypt revealed by UPGMA cluster analysis calculated from RAPD data.
TZ1 to TZ6 = isolates of *T. harzianum*; TH1 & 2 = two isolates of *T. hamatuum*; TG1 = one isolate of *T. glaucum*; TR1 = one isolate of *T. reesei*; TV1 = one isolate of *T. viride*.

The results obtained showed that biocontrol agent, *Trichoderma* spp., significantly reduced the root rot incidence and increased the number of survival plants (Tables 2 and 3). In general, all tested isolates of *Trichoderma* species tested significantly reduced the values of the disease severity indexes and increased the total number of surviving plants. The results obtained can be explained as follow:

A – *Fusarium solani:*

This pathogen was the most frequent fungus all over the different pathogens and was very aggressive showing high value of disease index (91.67). This pathogen was affected by *Trichoderma* spp. isolates which they were tested for their role as biocontrol of root rot incidence on the watermelon cv. Giza 1 in pot experiment. Results given in Table (2) showed that *Trichoderma* spp. were effective in reducing the incidence of the disease and increasing the total number of survival plants.

Table (2): Effect of different isolates of *Trichoderma* spp. as biocontrol agents against *F. solani*, one of the root rot fungal pathogens, on watermelon cv. Giza 1 in pots under greenhouse conditions.

Trichoderma spp.	Mean frequency of healthy and infected plants						Total Survivals		Disease [z] index
	Healthy (Type 0)[y]		Infected						
			Survivals (Type 1, 2, 3)		Dead (Type 4)				
	No.	%	No.	%	No.	%	No.	%	
T. harazianum									
TZ1	0.00	0.00	4.33	61.90	2.67	38.10	13	61.90	41.93
TZ2	0.670.	7.14	4.33	61.90	2.00	28.57	15	71.43	55.95
TZ3	000.0	0.00	6.67	95.24	0.33	4.76	20	95.24	50.00
TZ4	01.33	0.00	6.67	95.24	0.33	4.76	20	95.24	50.00
TZ5	2.00	19.05	3.67	52.38	2.00	28.57	15	71.43	55.95
TZ6		28.57	2.33	33.33	2.67	38.10	13	61.90	34.71
T. hamatum									
TM1	0.670.	9.52	2.33	33.33	4.00	57.14	9	42.86	69.05
TM2	67	9.52	2.00	28.57	4.33	61.90	8	38.10	73.81
T. glaucum									
TG1	0.00	0.00	4.00	57.14	3.00	42.86	12	57.14	67.86
T. reesei									
TR1	2.67	38.10	3.67	52.38	0.67	9.52	19	90.48	29.76
T. viride									
TV1	1.33	19.05	3.00	42.86	2.76	38.10	13	61.90	58.33
Check (*F. solani*)	0.00	0.00	2.33	33.33	4.67	66.67	7	33.33	91.67
L. S. D. at 5 %	0.31		0.26		0.45				0.88

[y] Infection Types (IT): 0 = no symptoms. 1 = few lesions (covering < 10 % of root), secondary root rot slight; 2 = rot of secondary roots or lesions covering approximately 25% of the root; 3 = lesions covering at least 50% of the root and dead secondary roots; 4 = general root rot, most of the root affected, also including pre and post emergence damping–off, (Aegerter *et al*., 2000).

[z] Diseas Index (D I) = [sum of (disease class × number of plants in class) x 100] ÷ [(total plants) × 4], (Kobriger *et al*., 1998).

The biological effect was clearly reflected on the values of disease index. However, there was some difference in the effect of the different species of the biocontrol agents as well as between different isolates of

species. Different isolates of *T. reesei*, *T. harzianum* and *T. viride* were the most effective ones in reducing the disease incidence and consequently the values of disease index. In general, all isolates of *T. reesei*, *T. harzianum* and *T. viride* reduced the values of disease index to more or less 50%. *T. reesei* TR1showed disease index value (29.76%) while, *T. harzianum* isolates showed disease index ranged from 34.71 to 55.95% and isolate of *T. viride* TV1 showed 58.33% respectively. Two isolates of *T. hamatum* and one isolate of *T. glaucum* were the least effective ones in reducing the amount of disease incidence and consequently the values of disease index. They showed high disease index values and low number of total survival plants.

B- *Rhizoctonia solani*: *R. solani*, the soil borne pathogen of root rot, was affected by some of the biocontrol agents. Generally, all isolates of different *Trichoderma* spp. tested were effective in reducing the amount of the disease compared with check treatment (pots inoculated with *R. solani* only). Isolates of *T. harzianum* were the most effective ones in reducing the disease index values which ranged from 36.90 to 55.95 and consequently increased the total number of survival plants. However, isolates of *T. hamatum*, *T. glacum*, *T. reesei* and *T. viride* were less effective which showed disease index more than 60%(Table,3).

Table (3): Effect of different isolates of *Trichoderma* spp. as biocontrol agents against *R. solani*, one of the root rot fungal pathogens, on watermelon cv. Giza 1 in pots under greenhouse conditions.

Trichoderma spp.	Mean frequency of healthy and infected plants						Total Survivals		Disease [z] index
	Healthy (Type 0)[y]		Infected						
			Survivals (Type 1, 2, 3)		Dead (Type 4)				
	No.	%	No.	%	No.	%	No.	%	
T. harazianum									
TZ1	0.670.	9.52	3.67	52.38	2.67	38.10	13	61.90	49.52
TZ2	001.3	0.00	5.67	80.95	1.33	19.05	17	80.95	55.95
TZ3	31.33	19.05	4.33	61.90	1.33	19.05	17	80.95	36.90
TZ4	0.330.	19.05	4.33	61.90	1.33	19.05	17	80.95	36.90
TZ5	00	4.76	5.00	71.43	2.00	28.57	16	76.19	55.95
TZ6		0.00	3.00	42.86	4.00	57.14	9	42.86	55.00
T. hamatum									
TM1	0.000.	0.00	3.00	42.86	4.00	57.14	9	42.86	69.05
TM2	67	9.52	4.33	61.90	2.00	28.57	15	71.43	60.33
T. glaucum									
TG1	0.33	4.76	4.00	57.14	2.67	38.10	13	61.90	61.24
T. reesei									
TR1	0.00	0.00	3.67	52.38	3.33	47.62	11	52.38	65.00
T. viride									
TV1	0.00	0.00	3.00	42.86	4.00	57.14	9	42.86	63.81
Check(*R. solani*,)	0.00	0.00	2.67	38.10	4.33	61.90	8	38.10	86.90
L. S. D. at 5 %	0.22		0.28		0.12				2.19

[y] **Infection Types (IT): 0 = no symptoms. 1 = few lesions (covering < 10 % of root), secondary root rot slight; 2 = rot of secondary roots or lesions covering approximately 25% of the root; 3 = lesions covering at least 50% of the root and dead secondary roots; 4 = general root rot, most of the root affected, also including pre and post emergence damping–off, (Aegerter *et al*., 2000).**

[z] **Diseas Index (D I) = [sum of (disease class × number of plants in class) x 100] ÷ [(total plants) × 4], (Kobriger *et al*., 1998).**

DISCUSSION

The most widely used biocontrol agents in the world are belonged to the fungal genus Trichoderma. *Trichoderma* spp. have been used with success against soil borne, seed borne, phyllosphere and storage rot diseases (Chet, 1987; Tronsmo and Hjeljord 1998 and Elad, *et al*., 1998). This has culminated in the commercial production of several *Trichoderma* species for the protection and growth enhancement of a number of crops in the United States (McSpadden Gardener and Fravel, 2002), and in the production of *Trichoderma* species and mixtures of species in India, Israel, New Zealand, and Sweden (Howell, 2003).

Trichoderma species have many advantages as active biocontrol agents against many fungi. They have rapid growth rate in culture, produce of numerous spores (conidia) and great arsenal of inducible polysaccharides-degrading enzymes. Thus, it can be propagated on a wide variety of carbon sources, so it is easy to find a reasonability cheap substrate for biomass production of the biocontrol agent (Amer, and El-Desouky, 1999). It is now possible to design media and growth conditions that stimulate these fungi to produce the type of biomass appropriate to the intend application (Tronsmo and Hjeljord 1998; Amer and El-Desouky, 1999 and Vinzant *et al*., 2001). Another advantage is the wide range of environmental conditions tolerated by the various *Trichoderma* species and isolates (Tronsmo and Hjeljord 1998).

In the present study, *T. harzianum*, *T. hamatum*, *T. glaucum*, *T. reesei* and *T. viride* were isolated from the rhizosphere of watermelon plants and from the surface of infected root tissues and differentiated on the basis of conidiophores patterns and conidial morphology described by Rifai, (1969) and Bissett (1991). Results obtained *In vitro* showed that all *Trichoderma* species reduced radial growth of the different pathogens in dual culture. *T. reesei* and *T. viride* isolates showed the highest percentage of inhibition reached more than 50% in many cases while *T. hamatum* and *T. glaucum* isolates showed moderate effect on the inhibition of the pathogens growth. Meantime, all *T. harzianum* isolates showed percentage of radial growth inhibition reached less than 50% with different pathogens.

DNA analysis obtained by RAPD technique in the identification and taxonomy of filamentous fungi was used with *Trichoderma* spp. In the present investigation results lead us to conclude that DNA based RAPD provide a reliable method for grouping isolates of *Trichoderma* spp. Also, there was a positive correlation between degrees of dark bands which appeared in the picture after staining according to RAPD technique

We are now testing the biocontrol ability of *Trichoderma* spp. isolates collected from different localities in Egypt with their reaction to root rot pathogens of watermelon and analyzing phyllogenetic similarities among isolates based on DNA pattern. These studies will provide a basis for evaluating the biocontrol ability of isolates specialization in *Trichoderma* spp. taking into account the impact of geographic isolation and distribution in relation to their DNA patterns. Moreover, Phyllogenitic relationship between isolates not related to their geographic origin or biocontrol ability.

RAPD markers made it possible to observe genetic variability in the DNA sequences. RAPD method offers great potential for generating large numbers of markers representing a random sample of the genome and has efficiently been used to give reliable and reproducible results for estimating the genetic variation.

The primer showed a high number of scorable bands. This number of fragments is expected because the number of different amplification products for each primer depends upon the primer sequence, the genomic sequence and the genome size.

Genomic DNA from two different individuals often produces different amplification fragment patterns. A particular DNA fragment which is generated for one individual but not for another represents a DNA polymorphism and can be used as a genetic marker.

The dendogram showed two clusters; the first one included the *T. harazianum* isolates. However, the second cluster included the *T. hamatum* isolates. This indicates the ability of the RAPD similarity technique to differentiate among *Trichoderma* species isolates according to their genetic similarity levels. This indicates that biocontrol ability of *Trichoderma* species isolates understudy not related with the genetic similarities among isolates. Therefore, it is important for the researcher to prescreen the genetic similarities among the *Trichoderma* species isolates before their involving in large scale programs of application.

The use of RAPD based-PCR to asses the genetic diversity and phylogenetic relationships between different *Trichoderma* species in relation to their variation in antagonistic ability was used by many investigators (Lexova *et. al.,* 1998; Muthumeenakshi *et. al.,* 1998; Yli-Mattila *et. al.,* 1999; Abbasi-Pervaiz *et al.,* 1999; Sivakumar *et al.*, 2000; Salama *et. al.,* 2002 and Maymon *et al.*, 2004).

Trichoderma species are active as hyperparasites. Generally, all species of *Trichoderma* species significantly affected the incidence of the disease in terms of the numbers of healthy and infected plants. Also, disease index values were assessed. *Trichoderma* spp. which significantly reduced the root rot incidence and increased the number of survival plants. Under greenhouse condition, the effectiveness of the boiagents was tested against the different pathogens using the susceptibe watermelon cultivar Giza 1. Results obtained showed the effectiveness of *T. harzianum* in this respect. Results obtained showed the superiority of *T. harzianum* isolates against the most of the tested pathogens and were varied in their effects on the seven pathogens. Successful biological control of soil borne pathogens by infesting the soil with cultures of *Trichoderma* spp. has been described (Wells *et al*., 1972 and Elad *et al*., 1981). Generally, the application of biocontrol agents reduced the incidence of root rot and increased the survival plants. *T. harzianum* were the most effective in biocontrol of different soil borne pathogens. Similar findings were obtained by (Kurzawinska, 1992; Zhao *et al*., 1998 and Ji *et al*., 2002).

It could be concluded that, *Trichoderma* species are easily isolated from the rhizosphere of rotted plants and from the surface of infected root. They are now present as soil borne fungi and established in the Egyptian soil

which affects the prevalence of many pathogens. In addition, the efficiency of its effect of these biocontrol fungi can be increased by increasing their population in the soil and / or selecting and applying the effective isolates which finally increase their efficiency against most pathogens. But, handling, carriers, storage, formulation and delivery system of these biocontrol agents remains the major problem in the application at large scale.

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توصيف أنواع الفطر تريكودرما باستخدام تحليل الحمض النووى وتفاعلها مع مسببات مرض عفن الجذور فى البطيخ

محمد أحمد عوض[1] ، شوقى محمد الدسوقى[2] ، ماجده محمد التويجرى[3] و شريف محمد الجنايني[2]

1- كلية الزراعة - جامعة المنوفية - شبين الكوم - مصر

2- معهد بحوث أمراض النبات - مركز البحوث الزراعية - الجيزه - مصر

3- كلية العلوم التطبيقية للطالبات - جامعة أم القرى - مكه المكرمة - المملكه العربية السعودية

يعتبر البطيخ من أهم محاصيل الخضر القرعية التي تزرع في مصر تحت ظروف الحقل المفتوح في العروة الصيفية لسد احتياجات المستهلك. وبالرغم من استخدام العديد من الأصناف عالية المحصول في الزراعة إلا أن الإصابة بالأمراض سواءً على الجذور أو المجموع الخضري تحد من الإنتاجية حيث تصاب نباتات البطيخ بالعديد من الأمراض الخطيرة واسعة الانتشار والتي قد تؤدى إلى موت النباتات وقلة عددها وبالتالي يؤدى الى نقص واضح في المحصول. وتركزت هذه الدراسة على مسببات أمراض عفن الجذور الفطرى ودراسة التطفل الفائق لفطريات التضاد الحيوي من جنس تريكودرما على هذه المسببات. وقد تلخصت نتائج هذه الدراسة فيما يلى:

تم جمع عينات نباتية مصابة من مناطق مختلفة شملت محافظات القليوبية, المنوفية, كفر الشيخ, البحيرة, الدقهلية، دمياط، المنيا، الأسماعيليه، شمال سيناء حيث تم عزل الفطريات الممرضة وكذلك الفطريات المصاحبة بالأضافه للفطريات من الجنس *Trichoderma* كائن التضاد الحيوى. أوضحت الدراسة عزل الفطريات:

Fusarium solani, Fusarium oxysporum f.sp. *nevium, Fusarium moniliforme, Monosporascus cannonballus, Fusarium equesti, Fusarium chlamydosporum, Rhizoctonia solani, Pythium aphanidermatum, Macrophomina phaseolina, Fusarium chlamydosporum* and *Trichoderma* spp., *Alternaria* spp., *Aspergillus* spp. and *Rhizopus* spp.

تم دراسة التضاد بين عزلات خمسة أنواع من جنس الفطر تريكودرما وهي: تريكودرما هارزيانم ، تريكودرما هاماتم ، تريكودرما جلوكم ، تريكودرما ريساى ، تريكودرما فيردى في المزارع المزدوجة على أطباق بترى ضد الفطريات الممرضه السبعه وهي:

F. solani, *R. solani, F. monoliforme*, *F.oxysporum* f. sp. *nevium*, *Monosporascus cannonballus, Macrophmina phaseolina,* and *Pythium aphanidermatum*

.وقد أوضحت النتائج المعملية تفوق الفطر تريكودرما هارزيانم يليه في ذلك الفطريات تريكودرما هاماتم ، تريكودرما جلوكم عن باقي الفطريات ضد غالبية الفطريات الممرضة المختبرة.

تم دراسة محتوي الحمض النووى DNA وقد إستخدم في هذه الدراسة عدد ١١ عزله من أنواع الفطر تريكودرما وأجري تحليل ودراسة الحزم الناتجة عن التفريد بطريقة RAPD إستخدم فيها البادئ المناسب لإظهار الفروق لإيجاد معلم Marker مرتبط بكفاءة هذه العزلات على التطفل الفائق والتضاد الحيوى . وقد أظهرت النتائج اختلاف العزلات عن بعضها البعض من حيث احتوائها على مناطق الاختلاف أو التشابة وقد أمكن الكشف عن المواقعً المتشابهة لعزلات كل نوع من أنواع الفطر تريكودرما وأيضا تم تطبيق أسلوب التحليل العنقودي لتصنيف هذه العزلات بناء على مابينها من درجة قرابة في أنماط DNA وتم التعبير عن النتائج في فينوجرام. وقد انقسم الفينوجرام إلى مجموعتين اشتملت المجموعة الأولى على عزلات الفطر تريكودرما ةهارزيانم والمجموعة الثانية على عزلات الفطر تريكودرما هاماتم . أما الفطريات تريكودرما جلوكم ، تريكودرما ريساى ، تريكودرما فيردى فقد وقع كل منها في مجموعه منفردة بعيدة القرابة عن المجموعات الأخرى. أظهرت الدراسه أن أنماط DNA المفكك بواسطة طريقة RAPD تصلح للتفرقه بين عزلات الفطر تريكودرما بناء على علاقة القرابه بين هذه العزلات و أن الاختلافات فى أنماط DNA بين العزلات الأحدعشر تحت الدراسه ليست مرتبطه بمنشاها الجغرافى أو قدرتها على التطفل الفائق والتضاد الحيوى.

وقد تم اختبارتأثير الأحدى عشرة عزله تحت الدراسه من فطر تريكودرما تحت ظروف الصوبة الزجاجية ضد بعض الفطريات الممرضه على صنف البطيخ القابل للإصابة (جيزة ١) وقد أوضحت النتائج كفاءة تلك الفطريات في عملية المقاومة الحيويه وأنه قد اختلفت جميع فطريات التضاد الحيوى في تأثيرها حسب الفطر الممرض مع تفوق عزلات الفطر تريكودرما هارزيانم و عزلات الفطر تريكودرما هاماتم يليه فى بعض الحالات عزلات الفطر تريكودرما ريساى.

Table (1): Radial growth and percentage of inhibition of seven watermelon root rot pathogens using eleven isolates of *Trichoderma* spp. in dual culture on PDA media.

***Trichoderma* spp.**	**Pathogens / Radial growth inhibition %[Y]**													
	***F. solani* RG [Z]**	**% inhibition**	***R. solani*** **RG**	**% inhibition**	***F oxysporum* f. sp. *nevium*** **RG**	**% inhibition**	***F.moniliforme*** **RG**	**% inhibition**	***M. phaseolina*** **RG**	**% inhibition**	***Monosporascus cannonballus*** **RG**	**% inhibition**	***P. aphanidermatum*** **RG**	**% inhibition**
T. harazianum														
TZ1	4.97	40.58	4.53	45.50	4.17	40.79	3.27	30.57	4.83	44.60	3.50	45.12	4.07	48.19
TZ2	4.97	41.65	4.53	45.67	4.00	39.17	3.63	38.26	4.90	42.21	4.13	42.74	4.10	47.07
TZ3	4.70	41.87	4.40	43.80	3.80	42.59	2.97	37.50	4.63	42.44	4.23	30.98	4.03	42.21
TZ4	4.77	39.44	4.20	38.94	4.17	27.76	3.07	57.64	4.37	46.00	4.77	36.94	4.10	37.45
TZ5	4.93	36.26	4.57	35.04	4.13	39.49	3.20	52.22	4.73	37.35	4.63	35.17	4.13	41.95
TZ6	5.03	41.79	4.47	41.05	3.20	45.70	3.07	61.95	5.20	38.53	4.20	41.26	4.07	37.72
T. hamatum														
TM1	4.80	35.45	4.13	47.62	4.13	46.92	3.00	54.30	4.63	47.63	3.70	43.28	3.93	40.02
TM2	4.30	40.89	4.03	48.01	4.00	49.21	2.73	56.05	4.53	47.13	4.30	37.88	4.03	38.78
T. glaucum														
TG1	5.50	28.64	4.33	41.08	4.37	44.26	3.97	48.76	5.30	35.26	3.83	48.70	3.83	41.07
T. reesei														
TR1	5.13	35.17	4.03	47.83	4.43	41.36	3.03	60.27	4.30	41.91	2.67	61.14	4.00	50.17
T. viride														
TV1	4.87	37.04	4.47	41.67	4.10	49.63	3.03	56.06	4.93	43.39	2.10	64.63	3.43	50.90
Control	9.00		9.00		9.00		9.00		9.00		9.00		9.00	
LSD at 5%	0.55		0.37		0.36		0.37		0.43		0.43		0.97	

[Y] Percentage inhibition of radial growth was calculated by $(R_1 - R_2) / R_1 \times 100$, where R_1 is the maximum radius of the pathogen colony and R_2 is the radius of that part of the pathogen colony directly opposite the biocontrol agent colony (Zhou and Reeleder, 1990).

[Z] Radial growth (cm) was measured when the control treatment covered the Petri dish.