Journal of Applied Pharmaceutical Science Vol. 5 (02), pp. 110-119, February, 2015
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2015.50216
ISSN 2231-3354

Studies on Fusarium wilt Disease of Cucumber

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ARTICLE INFO

Article history:
Received on: 25/11/2014
Revised on: 17/12/2014
Accepted on: 21/01/2015
Available online: 27/02/2015

Key words:
Cucumber, pathogenicity, *Fusarium*, biocontrol, *Trichoderma.*

ABSTRACT

Eight isolates of *Fusarium oxysporum f.sp. cucumerinum;* the causal organism of *Fusarium wilt of cucumber plants;* were isolated from wilted cucumber plant samples collected from eight different localities in Egypt . These isolates were pathogenic to the susceptible cucumber cultivar "*Biet alpha*". Isolate 3 was the most virulent, followed by isolate 4 , while the least pathogenic was isolate 5. Potato Dextrose medium was the best medium for linear and amount of growth for all tested isolates, whereas rate of growth varied between the different tested isolates with the different tested media. Optimum temperature for the fungal linear growth was 27°C, followed by 28°C, while the best amount of growth was obtained at 25°C for all tested isolates. Cucumber genotypes were susceptible to *Fusarium oxysporum f.sp. cucumerinum* and differed significantly in their susceptibility. The most tolerant cucumber genotype was *"Zeina"* as well as it recorded the highest survival plants. DNA analysis detected 33 loci with an average 4.125 loci/isolate. The highest number of polymorphic loci was 6 loci in both Fus 1&4 isolates, while the lowest was 1 loci in Fus 6 only. UPGMA cluster analysis divide into two sub clusters, one of them included Fus 6 only and the second include the rest seven isolates with different similarity values. 100 & 614 bp were detected in both highly pathogenic isolates and it could be carrying the genetic information affecting pathogenicity of *Fusarium* to cucumber plants. Grouping of *Trichoderma isolates* in UPGMA cluster analysis and variation in DNA profiles among isolates was not related to their biocontrol ability. Biocontrol agents great affected *Fusarium* growth in dual cultures and reduced it under laboratory conditions. Biocontrol agents minimized the disease parameters in comparing with infested control treatments by the most aggressive isolate under greenhouse condition.

INTRODUCTION

Three Genera of cucurbits *i.e*. *Cucumis* spp. (cucumber & melon), *Citrullus* spp. (watermelon) and *Cucurbita* spp. (squash & pumpkin) are widely grown warld wide. They can be grown in different seasons throughout the year round, in open fields and in protected cultivations. Cucumber (*Cucumis sativus* L.) is considered one of the major vegetable summer crops in commercial fields in Egypt. However, during the last few decades efforts were concentrated to grow the crop in protected system in greenhouses during autumn and winter seasons. The cultivated area of cucurbits in Egypt is progressing at a relatively fast rate, especially in newly reclaimed desert lands. Several fungal diseases attack cucumber during different growth stages causing considerable losses in fruit yield. These diseases are downy mildew, powdery mildew, gray mould, root rot, white mould, Fusarium wilt, gummy stem blight, anthracnose, and black rot. Soil borne diseases are economically very important and responsible of losses in fruit yield due to diseases infection. *Fusarium oxysporum* f. sp. *cucumerinum* is the most common pathogen on cucumber plants causing Fusarium wilt on cucumber and reduced the yield (Ogura *et al.*, 1992; Martinez, *et al.* 2003). Also, Soil borne pathogenic fungi i.e. *Rhizoctonia solani*, *Pythium* spp., *Phytophthora* spp., *Macrophomina phaseolina* and *Sclerotium rolfsii* are the most common pathogens associated on cucumber plants causing damping-off and root rots to cucumber (Bedlan, 1986). *Fusarium oxysporum* f. sp. *cucumerinum* was isolated from the infected roots of cucumbers and recorded in many areas (Huang, 1990; Huang *et al.* 1994). Michail *et al.*, (1989) found that *Fusarium oxysporum* f.sp. *cucumerinum* and *F. oxysporum* f.sp. *niveum* were to be externally and internally seed borne in cucumber and watermelon, respectively. Each pathogen caused severe symptoms of wilt on the affected plants. Ogura.

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(1992**)** studied the dispersal and survival ability of *Fusarium oxysporum* f.sp. *cucumerinum* in soil at Japan. He reported that the pathogen in plant debris survived more than 1 year in soil, but the propagules on their own survived only 4 months. Chen Mine *et al*. (2003) mentioned that the change in the roots, stem base and stem of seedlings of resistant and susceptible cucumber cultivars inoculated with *Fusarium oxysporum* f.sp. *cucumerinum*, as well as the mycelium growth of the fungi in the crop were studied. Murakami *et al.* (2003) found that the effects of the cultivation of preceding plants on the inhibition of fusarium wilts, vessel disease of cucumber (*Cucumis sativus*) caused by *Fusarium oxysporum* f.sp. *cucumerinum* (FOC). Using RAPD based-PCR to assess the genetic diversity and phylogenetic relationships between different *Fusarium oxysporum* isolates to distinguish the variation in pathogenicity ability of the different *Fusarium oxysporum* isolates were reviewed by many investigators. Welsh and McClelland (1990) reported that random amplified polymorphic DNA could provide simple and reproducible fingerprints of germplasm by employing single, arbitrary chosen primers. Vakalounakis and Fragkiadakis (1999**)** found that a total of 106 isolates of *Fusarium oxysporum* obtained from diseased cucumber plants showing typical root and stem rot or *Fusarium* wilt symptoms were characterized by pathogenicity, vegetative compatibility, and random amplified polymorphic DNA (RAPD). Vakalounakis, *et al.,* (2004) found that thirty-four isolates of *Fusarium oxysporum*, obtained in China from cucumber plants showing either *Fusarium* wilt (*F. oxysporum* f.sp. *cucumerinum*) or root and stem rot (*Fusarium oxysporum* f.sp. *radicis-cucumerinum*) symptoms, were characterized by pathogenicity, vegetative compatibility and randon amplified polymorphic DNA (RAPD). The use of RAPD based-PCR to assess the genetic diversity and phyllogenetic relationships between different *Trichoderma* spp. isolates to distinguish the variation in antagonistic ability of the different *Trichoderma* spp. isolates were reviewed by many investigators. Maymon *et al.* (2004) identified a collection of *Trichoderma* species isolates, which differed in their biocontrol capabilities, by molecular methods. Lifshitz *et al*., (1986) explained several factors precluded mycoparasitic interactions of *Trichoderma harzianum* or *T. koningii* isolates to *Pythium* sp. and *Fusarium oxysporum* f.sp. *cucumerinum*. Such interactions were observed infrequently and occurred at 24 h or more after mycelial contact in dual culture. Germination of *Trichoderma* conidia required > 10-14 h of incubation at 26°C. Rose *et al*., (2004) found that the biological control agents provide a potential means of disease control if efficacy data were available to support their use. Several commercially available biological control agents were evaluated for their ability to reduce disease due to Fusarium and Pythium root rots on cucumbers. Therefore, the aim of the present investigation was to study the common and frequent isolates of Fusarium wilt pathogen attacking cucumber plants . Also, electrophoresis studies (Polymerize Chain Reaction, PCR) were carried out on DNA patterns of different *Fusarium oxysporum* f. sp. *cucumerinum* isolates as well as biological control agent isolates. To avoid the hazards of using the fungicides, an alternative control method was studied using biological control agents against the pathogen *in vitro* and *in vivo*.

MATERIALS AND METHODS

This work was started by collecting naturally infected cucumber plants showing wilt symptoms from root rotted seedlings and plants, and from damped-off seedlings. Isolation of the causal organism and the associated fungi was done from the diseased samples in two successive seasons. Pure cultures were obtained by single spore technique and/or selecting hyphal tips of the non-sporulating fungi. The pure cultures of the growing fungi of the causal organism and the associated fungi were then examined microscopically and identified according to the methods adopted by Neergard (1945), Barnett (1960), and Domsch *et al.,* (1980). The Dilution Plate Method (DPM) was used for the isolation of the biological control agents. Soil and rhizosphere samples were taken from the same cucumber fields by uprooting the infected plants with great care to obtain most of the intact root systems. . Serial dilutions were made to study the micro flora, according to the method proposed by Beninashemi and Dezeeuw (1969). Martin‘s medium at dilution of 10^{-5} was used for the fungal count as described by Martin (1950). Trichoderma Selective Medium (TSM) was used for isolation of *Trichoderma* species isolates from soil (Elad *et al.,* 1981). Isolates of *Trichoderma* species obtained from the rhizosphere of cucumber plants were identified after growing them on 20% malt extract agar in plates, which were incubated for two days at 25°C. All isolates were then examined microscopically and identified according to the method adopted by Rifai (1969) and Bissett (1991). Phathogenicity tests were conducted using eight isolates including fungi obtained from eight localities of six governorates. These fungal isolates were identified previously as isolates of *Fusarium oxysporum* f.sp *cucumerinum*. These fungal isolates were tested for their pathogenicity on the commercial cucumber cultivar “Beit alpha”. Inoculums were prepared using barley grain + corn meal + wheat bran medium. Disease incidence was recorded as number and percentages of pre-emergence damping-off (two weeks after sowing), post-emergence damping-off (four weeks after sowing) and number of survival plants (Sixty days after sowing). wilt disease severity index was estimated after sixty days from planting according to Soliman *et al.,* (1988**)**, modified by Awad (2004) .

Disease severity index was estimated according to the type of discoloration observed internal roots of inoculated plants .The disease index was calculated using the following equation: Disease index (DI) = [Sum of (disease grade x number of plants in grade) 100] ÷ [(No. of total plants)X maximum of grade infection (4)]. Eleven cucumber genotypes i.e., Medina, Zeina, Sweet crunch, Mena, Thamin, Beit alpha, Seina 2, Shorouk, El-Hout, Samara and Melita were evaluated against the most virulent isolate No. 3 under greenhouse conditions. Eight isolates of *Fusarium oxysporum* f.sp *cucumerinum* were tested for DNA analysis *in vitro*, as well as six isolates of biological control agents (*Trichoderma spp*) were also chosen for the same analysis to

explain the pathogenicity and bioaction among all isolates of both pathogenic and bioagent fungi. DNA isolation from *Fusarium oxysporum* f.sp *cucumerinum* and *Trichoderma* spp. Isolates was performed according to the procedures of Al-Samarrai and Schmid (2000).

For fungal growth synthetic complete medium of Anjani and Panda (1995). 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' . 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. Data analysis was conducted using the Numerical Taxonomy and Multivariate Analysis system, Version 2.1 (NTSYSpc; Rolhf, 2000). 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.

The reaction between three isolates from all the isolates of *Fusarium oxysporum* f.sp *cucumerinum* (the most virulent two isolates **(**F3 & F4**)** and the least virulent isolate **(**F5**)** and six biological control agents i.e. *Trichoderma harzianum* (Th1& Th2), *T. ressei*, *T. viride*, *T. hamatum* and *T. glaucum* was carried out in dual cultures under laboratory conditions , all biological actions were recorded i.e. inhibition zone and/or over growth. Six isolates of *Trichoderma* spp were selected for study the biological control under greenhouse conditions. All data obtained were subjected to the proper statistical analysis for each experiment using the MSTAT statistical software. Comparisons were made following Fishers LSD (0.05).

RESULTS AND DISCUSSION

Fusarium oxysporum f.sp. *cucumerinum* attacks cucumber plants at any stage of plant growth, pre and post-emergence damping-off may occur; wilt symptoms develop first on lower and middle leaves and then spread to the top of plants. Early infection of plants prevented fruit set, while later infection resulted in small, abnormal fruits. Cracks often appear on diseased vines. Vascular discoloration of the roots and stems was noticed. The central part of the taproot shows a deep red.

Isolation of the causal organisms

Eight isolates of *Fusarium oxysporum* f.sp. *cucumerinum* were isolated from wilted cucumber plant samples collected from eight different localities in Egypt. *Fusarium oxysporum* f. sp. *cucumerinum* is the most common pathogen on cucumber plants causing Fusarium wilt on cucumber and reduced the yield (Ogura, 1992; Martinez, *et al*. 2003).

Pathogenicity tests

Eight isolates of *Fusarium oxysporum* f.sp. *cucumerinum* were chosen for tested their virulence against the susceptible cucumber cultivar Beit alpha. Data present in table (1) show that all tested isolates of fungi were pathogenic to cucumber Beit alpha cultivar. Isolate No. 3 was the most virulent isolate (aggressive), (it recorded % disease index (DI) (77.33%), followed by isolate No. 4 (74.66%). The least virulent isolate that gave the lowest percentage of disease index was isolate No.5 (57.33%) followed by isolate No. 2, (62.66%). Significant differences were noticed between the tested 8 isolates and they were varied among percentages of disease index as compared with control. The highest percentage of pre-emergence damping-off was noticed in cucumber plants inoculated with either No. 3 and No. 4 isolates, (26.66%), followed by isolate No. 8, (21.33%), whereas the lowest pre-emergence damping-off was recorded by isolate No. 5, (10.66%), followed by isolates No. 1, 6 and 7 (13.33%). The lowest post emergence damping-off was recorded on Biet alpha cucumber cultivar with *Fusarium* isolate No. (5) (5.33%), followed by isolates No. 2 and 7 (7.99%), whereas the highest value in the same parameter was recorded by isolate No. (1) (15.99%), followed by isolates 3 and 4 (13.33%) (Table 1).

These data indicate also that the highest percentage of survival plants were recorded in Biet alpha cucumber plants that inoculated with isolate No. 7, it was 95.99%, followed by isolate No. 1 (82.66%) which recorded the second rank in survival plants. The lowest percentage of survival plants was in cucumber plants infested with isolate No. 4, it was 58.66%, followed by cucumber plants inoculated with isolate No. 3 (61.33%). These results indicate also that no significant differences between all inoculated cucumber plants (8 isolates) in their shoot fresh weight of the commercially grown cucumber genotype "Beit alpha" plants. It could be concluded from the above results that obtained from pathogenicity tests that, the most aggressive or virulent isolate between all eight tested isolates of *Fusarium oxysporum* f.sp. *cucumerinum* was isolate No.3 , followed by isolate No.4, while the weakest pathogenic isolate was isolate No.5 . These results are in accordance with previous studies carried out on the pathogenic and genetic characterization of *Fusarium sp* **(**Sidorova and Akmuradov, 1980; Palmer and Williams, 1981; Pelcz, 1984; McMillan, 1986; Huang, 1990 ; Lakhdar, *et al.*, 2004).

Varietal Resistance

The response of eleven cucumber genotypes to wilt infection incited by *Fusarium oxysporum* f.sp. *cucumerinum* was studied under greenhouse conditions i.e. Medina, Zeina, Sweet Crunch, Mena, Thamin, Biet alpha, Seina 2, Shorouk, El-Hout, Samara and Melita were tested against the most aggressive isolate *of Fusarium oxysporum* f.sp. *cucumerinum* (No.3) for studying their response (resistance and susceptibility) as well as growth parameters as affecting by wilt incidence and infection.

Table 1: Virulence of eight isolates of *Fusarium oxysporum* f.sp. *cucumerinum* on cucumber cultivar " Biet alpha" under greenhouse conditions.

Isolate No.	Disease parameters %			Growth parameters (gm fresh weight)		Survival plants%
	Pre-emergence	Post-emergence	Disease index D.I.	Shoot	Root	
1	13.33	15.99	70.00	33.99	2.50	82.66
2	15.99	7.99	62.66	37.02	2.79	74.66
3	26.66	13.33	77.33	34.95	1.85	61.33
4	26.66	13.33	74.66	39.84	2.23	58.66
5	10.66	5.33	57.33	40.67	3.05	79.99
6	13.33	7.99	65.77	38.60	2.75	69.33
7	13.33	00.00	72.88	35.67	2.65	95.99
8	21.33	10.66	65.33	35.39	2.91	63.99
Control	0.00	0.00	16.41	60.84	3.60	100.00
LSD at 5%	11.36	5.85	7.87	12.74	0.87	17.89

Table 2: Response of eleven cucumber genotypes to wilt infection incited by the most aggressive *Fusarium oxysporum* f.sp. *cucumerinum* isolate under greenhouse conditions.

Cucumber genotype	*Fusarium oxysporum* f.sp. *cucumerinum* (Isolate No. 3)					
	Disease parameters %			Survival plants%	Growth parameters (fresh weight gm)	
	Pre-emerge.	Post-emerge.	*Disease Index*		Shoot	Root)
Medina	35.00	40.00	88.33	25.00	41.46	2.00
Zeina	20.00	15.00	76.66	65.00	71.30	1.68
Sweet crunch	25.00	50.00	91.66	25.00	58.91	2.10
Mena	50.00	25.00	90.00	25.00	68.94	2.50
Thamin	40.00	25.00	90.00	35.00	61.32	1.76
Beit alpha	55.00	30.00	98.33	15.00	36.39	1.17
Seina 2	25.00	30.00	85.00	45.00	45.68	2.20
Shorouk	55.00	25.00	92.50	20.00	58.83	2.04
El-Hout	60.00	20.00	92.50	20.00	46.07	1.49
Samara	30.00	25.00	90.00	25.00	51.27	1.58
Melita	45.00	40.00	95.00	15.00	34.00	1.06
L.S.D. at 5%	12.23	17.81	18.64	12.11	15.02	0.24

Data in Table (2) indicate that all cucumber genotypes tested were susceptible to *Fusarium oxysporum* f.sp. *cucumerinum* and differed significantly in their susceptibility against *F. oxysporum* infection. All cucumber genotypes were varied in pre emergence damping-off incidence and significant differences were noticed between all tested cucumber genotypes; the most susceptible variety was El-Hout, it was reacted with 60% followed by Shorouk and Biet alpha (55%). The least susceptible one to pre-emergence damping-off was Zeina (20%), followed by sweet crunch and Seina 2 (25%). The rest cucumber genotypes were reacted with values between the most and least susceptible varieties. These data indicate also that, all rested cucumber genotypes were fairly susceptible to post-emergence damping-off and showed some significant differences in their susceptibility.

The least susceptible variety to post-emergence damping-off was Zeina (15.00%), followed by El-Hout variety (20.00%), while the most susceptible one was sweet crunch (50.00%), followed by Medina and Melita genotypes (40.00%). The rest genotypes were fall in between. Regarding Table (2), the data presented indicate also that all cucumber genotypes tested were susceptible to isolate (3) of *Fusarium oxysporum* f.sp. *cucumerinum*, which differed significantly on its effect on cucumber genotypes. All cucumber genotypes were reacted and differed significantly in their susceptibility against *F. oxysporum* infection. Disease severity index (DI) was recorded the highest DI on "Beit alfa" genotype as affected by *Fusarium oxysporum* f.sp. *Cucumerinum* isolate (3) (the most aggressive isolate), followed by Melita (98.33 and 95.00%, respectively). The least DI was recorded by Zeina genotype, followed by Seina 2 genotype (76.66 and 85.00%, respectively). Data in Table (2) also indicate that the highest surviving plants was recorded in the least susceptible cucumber genotype "Zeina" against the tested aggressive fungal isolate No.3, followed by "Seina 2" (65.00 and 45.00%, respectively). The least survival plants was recorded by "Biet alpha" and "Melita", followed by "El-Hout" and "Shorouk" genotypes (15.00 and 20.00%, respectively). Generally, the percentage of surviving plants was closed opposite correlated with pre-and post-emergence damping-off. The growth characters of cucumber genotypes that planted in soil infested with the most aggressive isolate (No. 3) of *Fusarium oxysporum* f.sp. *cucumerinum* had affected by wilt infection under greenhouse conditions. Significant differences were found between root fresh weights and shoot fresh weights obtained data. "Zeina" cucumber genotype recorded the highest shoot fresh weight (71.30 gm) followed by "Mena" genotype (68.94 gm).

The least shoot fresh weight was recorded by "Melita" genotype, followed by "Beit alpha" cucumber genotypes (34.00 and 36.39%, respectively). The most root fresh weight was recorded by "Mena" genotype (2.50 gm) followed by "Seina 2" cucumber genotype (2.20 gm), whereas the least root fresh weight was recorded by "Melita" followed by "Biet alpha" cucumber genotypes (1.06 and 1.17 gm, respectively). Similar results on cucumber varieties and genotypes were reported by many investigators (Vakalounakis, 1995; Dyakunchak and Ostroukh, 1998; Punga and Parker, 2000; Murakami *et al.*, 2003; Buriev *et al.*, 2004).

DNA analysis

In the present work, random amplified polymorphic DNA analysis obtained by electrophoretic techniques was used. Also, computerized program for cluster analysis to differentiate among DNA profiles of eight *Fusarium oxysporum* f.sp. *cucumerinum* isolates obtained from wilted cucumber plant samples collected from different localities in Egypt, as well as six of *Trichoderma* species isolates obtained from the rhizospher of cucumber roots grown in the same different localities in Egypt. This electrophoretic banding pattern of both fungal pathogen and bioagent species isolates RAPD are shown in (Figs.1 &3).

Random amplified polymorphic DNA (RAPD) analysis of Fusarium spp. isolates

Genomic DNA prepared from the eight *Fusarium oxysporum* f.sp.*cucumerinum* isolates namely; (Fus 1 to Fus 8); 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 sample were scored for presence and absence of amplification products. The primer was chosen to screen the eight Fusarium isolates, had proven to detect polymorphism in the absence of specific nucleotide in the fungal pathogen hyphae.

This primer is widely used in the RAPD experiments. The primer showed total number of scorable bands (33 bands) (Table 3) and (Figs. 1&2). The primer detected 33 fragments where their sizes ranged from 34 bp to 2932 bp (Table3). All of them were polymorphic (100%). The number of detected bands by this primer was high and the number of effective polymorphic fragments was high too.

To compare the polymorphism among these fungal isolates using the obtained RAPD markers, the total number of identified loci and the numbers of polymorphic loci were calculated. The polymorphic locus is the locus, which showed different pattern (presence or absence) in any isolate. The data is summarized in Table (3).

The total number of loci detected was 33 with an average of 4.125 loci per isolate. The results indicated that the highest number of polymorphic loci obtained was in both Fusarium isolates (Fus 1 & Fus 4) and the lowest numbers was in Fusarium isolate (Fus 6) with an average of 4.125 loci per isolate.

UPGMA cluster analysis divided the eight Fusarium isolates into two clusters. The first cluster included seven Fusarium isolates, i.e. Fus 1, Fus 2, Fus 3, Fus 4, Fus 5, Fus 7, and Fus 8. The second cluster included one Fusarium isolate (Fus 6) only. Generally, the second cluster included one of the moderate pathogenic isolate (F6), this isolate was not related to the others in the first sub cluster and showed low similarity (41.08%). The first sub cluster divided into two sub sub-clusters with low similarity (55.05%), one of them included three Fusarium isolates and the second included 4 isolates. One sub sub-cluster included one moderate pathogenic isolate only (Fus 1). The second sub sub-cluster that included the highly and low pathogenic isolates (Fus 3 & Fus 5, respectively) showed high similarity between them (93.51 %). The similarity between (Fus 1) and (Fus 3&4) showed (81.66%).

The similarity value between the three moderately pathogenic Fusarium isolates (Fus 2, Fus 7 and Fus 8) and the highly pathogenic one (Fus 4) was 73.48%, while it exceeded to 97.58% similarity between Fus 7 & Fus 8, both Fusarium isolates were in similarity with Fus 2 (80.81%) in one sub sub- cluster. Only one DNA fragment (1193 bp) was detected in the isolate Fus 6. The lowest pathogenic isolate (Fus 5) include three DNA fragments (2304, 1821 & 759 bp), whereas the remaining 29 DNA fragments were detected in the six Fusarium isolates tested. As the DNA fragments of 2256 bp, 1724 bp, 1000 bp, 807 bp, 614 bp and others were the common detectable bands between the six highly and moderately pathogenic isolates.

Four DNA fragments (1775, 1000, 614, & 324 bp) were detected in the highly pathogenic Fusarium isolate (Fus 3), two DNA fragments of them (100 & 614 bp) were detected in the other highly pathogenic isolate (Fus 4). It could be carrying the genetic information affecting the pathogenicity virulence of *Fusarium oxysporum* f.sp. *cucumerinum* to cucumber plants.

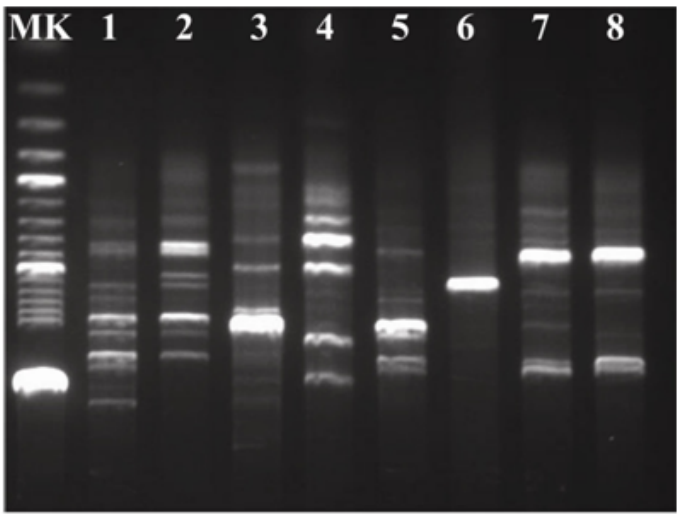

Fig. 1: Polymorphic DNA bands within eight *Fusariun oxysporum* f.sp. *cucumarinum* isolates amplified with random primer and electrophoresed in 1% agarose gel.

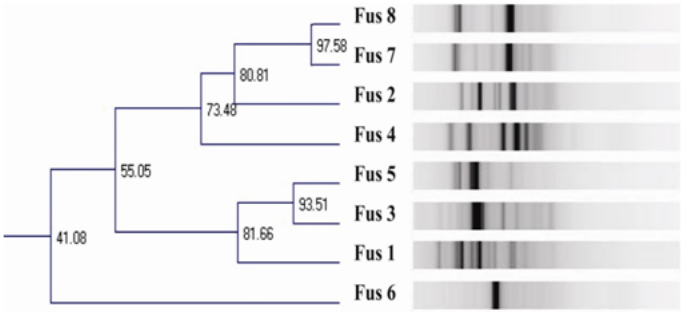

Fig. 2: Dendrogram showing a cluster analysis of eight *Fusariun oxysporum* f.sp. *cucumerinum* isolates based on polymorphism of 33 RAPD marker

Table 3: The presence and absence of thirty-three DNA fragments in eight *Fusariun oxysporum* f.sp. *cucumarinum* isolates.

Bp	Fusariun oxysporum f.sp. cucumarinum isolates							
	1	2	3	4	5	6	7	8
2932	+							
2545				+				
2401							+	
2304					+			+
2256	+	+						
2014				+				
1918	+							
1821					+		+	
1775			+					
1724	+	+						
1338							+	
1290	+							+
1193						+		
1097		+						
1000			+	+				
807							+	+
759	+				+			
710		+						
614			+	+				
442			+					
324		+		+				
179							+	
34				+				
Sum	6	5	4	6	3	1	5	3

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

Genomic DNA prepared from the six 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 sex *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 3).

To illustrate the genetic distance among *Trichoderma* species isolates, a phylogenetic tree was obtained using UPGMA clustering method. The dendrogram (Fig 4) 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. (4). The dendrogram arranged isolates of *T. harzianum* that they belonged to remotely related one sub cluster. Also isolate of *T. hamatum* and *T. glaucum* belonged to relate another sub cluster, while the isolates of *T.viride* and *T. reesei* were not related to them and showed low similarity to the two sub clusters mentioned before. The dendrogram was divided into two-sub cluster (SL = 53.40). The two isolates of *T. harzianum* were more closely related and showed higher similarity (65.37%), which formed a single sub cluster, while the isolates of *T. hamatum* and *T. glaucum* formed the another sub cluster with similarity (63.25%). The isolates of T. viride and T. reesei seemed to be not related to them. But T. reesei showed low similarity to the two sub clusters mentioned before (SL = 39.83%), while, isolate T.viride showed very low similarity to other all isolates (SL = 34.56%). The observed variations among the analyzed isolates show that grouping of Trichoderma species isolates in these sub clusters were related to their morphological characters and identification.

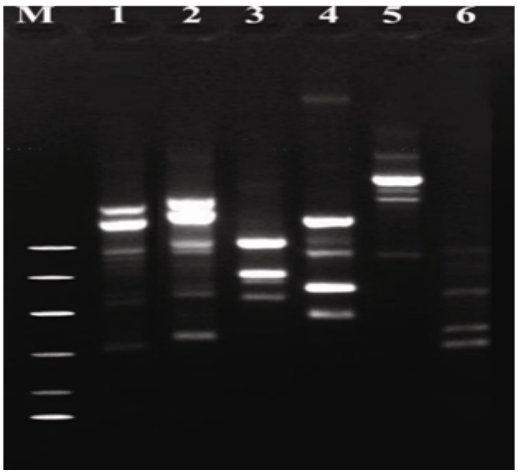

Fig. 3: Polymorphic DNA bands within six *Trichoderma* spp. isolates amplified with random primer and electrophoresed in 1% agarose gel.
M=DNA marker, 1 & 2 = isolates of *T.harzianum* (TZ1&2), *3*= isolate of *T.hamatum* (TH), 4= isolate of *T. reesei* (TR), 5= isolate of *T.viride (*TV), and 6= isolate of *T.glaucum (TG)*

Also, the observed variation in DNA profiles among isolates was not related to their biocontrol ability. These results led to conclude that the differences in DNA profiles among isolates of *Trichoderma* species were not related to biocontrol ability. Similar results were obtained on *Fusarium oxysporum* f.sp. *cucumerinum* on cucurbits (Vakalounakis and Fragkiadakis ,1999; Smith *et al.,* 2001; Wang *et al.,* 2001; Vakalounakis, *et al.,* 2004; Ninet, *et al.,* 2005). Williams, *et al.* (1990) reported that random amplified polymorphic DNA markers can detect a large number of genetic polymorphism and when linked to major genes can be potentially useful in identifying traits. The method of generating molecular markers by the RAPD technique amplifies short DNA sequences of the genome of an individual Random amplified polymorphic DNA markers could also be used in monitoring diversity within plant populations**.** Levi and Rowland (1997) mentioned that some findings suggest caution in making conclusions regarding genetic relationships of cultivars or selections within species and question the reproducibility random amplified polymorphic DNA markers**.**

Lexova *et. al.,* (1998**)** determined whether random amplified polymorphic DNA (RAPD) techniques to characterize *T. virens* rapidly and reliably on *T. virens* (3 isolates) and *T. roseum*. They reported that dendograms were constructed for each single primer pair and all the strains were clearly distinguishable by each single primer. They suggested that the simplified RAPD analysis could be used for the rapid identification of strains.

The observed variations among the analyzed isolates show that grouping of Trichoderma species isolates in these sub clusters was related to their morphological characters and identification. Also, the observed variation in DNA profiles among

isolates was not related to their biocontrol ability. Similar results were obtained by Salama *et. al.*, (2002) and Maymon et al. (2004), These results led us to conclude that the differences in DNA profiles among isolates of Trichoderma species were not related to biocontrol ability.

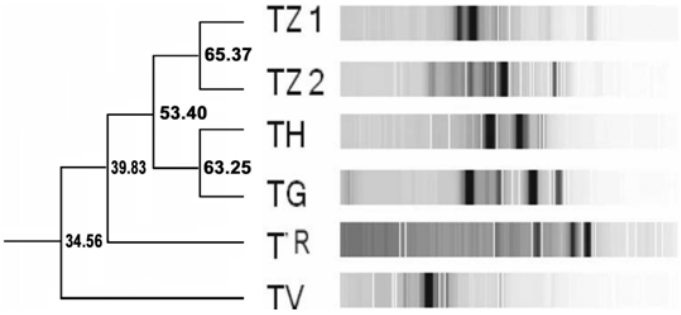

Fig. 4: Dendrogram showing analysis of six *Trichoderma* spp. Isolated from rhizosphere of cucumber roots grown in different localities in Egypt revealed by UPGMA cluster analysis calculated from RAPD markers data.

Biological control

Antagonistic activity of some biological control agents under laboratory conditions

The effect of some biological control agents on growth of three isolates of *Fusarium oxysporum* f.sp. *cucumerinum* i.e. (the most virulent two isolates; (No. 3 and 4) and the least virulent one (isolate No.5) were studied in Petri dishes on PDA medium under laboratory conditions. Six isolates of *Trichoderma* spp. i.e. *T. harzianum*1 (Th1), *T. harzianum*2 (Th2), *T. hamatum, T. glaucum, T. ressei* and *T. viride,* were used for determination the antagonistic activities against *Fusarium oxysporum* f.sp. *cucumerinum* (three isolates) in dual culture on PDA medium under laboratory conditions. Three parameters were measured for antagonistic activities in Petri dishes under laboratory conditions, i.e. linear growth, reduction of fungal growth under bioagent stress and over growth and/or inhibition zone. Data presented in Table (4) indicate that biological control agents greatly affected Fusarium growth in dual cultures. Significant differences between linear growth of the different pathogenic isolates. *T. reesei* greatly affected linear growth of isolate No. 3, followed by *T. viride,* whereas the least effect of the bioagents was noticed by *T. glaucum* against the same pathogen isolate (the linear growth was 35.50, 40.30 and 61.50 mm, respectively. The highest percentage of growth reduction of the same pathogen isolate was recorded by *T. reesei,* followed by *T. viride*, while the lowest growth reduction was recorded by *T. glaucum* (60.55, 55.22 and 31.66%, respectively).*Trichoderma. reesie* was grown over the pathogen fungal growth (isolate 3) with long distance (31.30 mm), whereas *T. hamatum* and *T. glaucum* contacted the pathogen isolate growth and not reacted with them (no inhibition zones or over growth were noticed). Other three bioagents were reacted with the pathogen isolate with inhibition zones. *T. viride,* Th1 and Th2 were inhibited mycilial growth of the isolate No 3 of *Fusarium oxysporum* f.sp. *cucumerinum*, the inhibition zones were 2.00, 1.30 and 2.60 mm, respectively. Data in Table (4) show also that, linear growth of Fusarium isolate No. 4 was inhibited at the least diameter by *T. harzianum* (Th1)(32.30 mm) followed by *T. hamatum* (34.50 mm), whereas the rest fungal biological control agents had less effect on the pathogen fungal isolate No. 4. The highest growth reduction was recorded by *T. harzianum* isolate 1 (Th1), followed by *T. hamatum*, while the least one was recorded by Th2 (64.11, 61.66 and 48.88%, respectively). Th1 and *T. hamatum* were contacted with the fungal growth of Fusarium isolate 4 without inhibition zone or over growth. *T. reesei* and *T. viride* were inhibited mycelial growth of the pathogen isolate 4 and the most inhibition zone was recorded by *T. viride* (2.30 mm). Biological control agents affected linear growth of Fusarium (isolate 5) also in dual culture. *T. viride* was the most effective one (33.50 mm), followed by *T. harzianum* 1 (TH1) (38.10 mm), while the least effective bioagent was *T. glaucum*, followed by *T. reesie* (56.60 and 56.10 mm, respectively). The maximum growth reduction was recorded by *T. viride* (62.77%), followed by Th1 (57.66%), while the minimum growth reduction was recorded by *T. glaucum* (37.11%), followed by *T. reesie* (37.66%) (Table 4).

T. reesie and *T. hamatum* were grown over pathogen mycelium (Isolate 5) and they recorded 35.60 and 12.30 mm, respectively. Th1 and Th2 were inhibited mycelial growth of the pathogen isolate 5 and the inhibition zones were 3.60 and 3.80 mm, respectively. The rest two bioagents were contacted the mycelial growth of the pathogen isolate without inhibition zone or over growth reactions. These results are in accordance with those obtained by Barnett and Binder (1973), Zhao *et al*., (1998), Howell (2003) , and Awad (2004). Dennis and Webster (1971 a and b) reported that, *Trichoderma* spp. differed in their antibiotical activities. They tested nine isolates of *Trichoderma harzianum* in their ability to produce antifungal antibiotics and they found that, some isolates inhibited *Fusarium annosus***.**

Table 4: Effect of some biological control agents on growth of three isolates of *Fusarium oxysporum* f.sp. *cucumerinum* under laboratory conditions.

Bioagent	Fungal isolate											
	3				4				5			
	Linear growth (mm)	Growth reduction (%)	Bioaction (mm) Over growth	Bioaction (mm) Inhibition zone	Linear growth (mm)	Growth reduction (%)	Bioaction (mm) Over growth	Bioaction (mm) Inhibition zone	Linear growth (mm)	Growth reduction (%)	Bioaction (mm) Over growth	Bioaction (mm) Inhibition zone
T. reesei	35.50	60.55	31.30	- - - -	36.00	60.00	- - - -	1.00	56.10	37.66	35.60	- - - -
T. viride	40.30	55.22	- - - -	2.00	35.10	61.00	- - - -	2.30	33.50	62.77	- - - - -	- - - -
T. harzianum (Th1)	42.30	53.00	- - - -	1.30	32.30	64.11	- - - -	- - - -	38.10	57.66	- - - -	3.60
T. hamatum	43.60	51.55	- - - -	- - - -	34.50	61.66	- - - -	- - - -	46.50	48.33	12.30	- - - -
T. harzianum (Th2)	43.60	51.55	- - - -	2.60	46.00	48.88	- - - -	2.00	47.00	47.77	- - - -	3.30
T. glaucum	61.50	31.66	- - - -	- - - -	43.60	51.55	- - - -	2.00	56.60	37.11	- - - -	- - - -

Biological control studies under greenhouse conditions

Six biological control agents i.e. *Trichderma reesie, T. viride, T. harzianum* 1 (Th1), *T. harzianum* 2 (Th2), *T. hamatum,* and *T. glaucum* were used for study their effect against three isolates of *Fusarium oxysporum* f.sp. *cucumerinum* i.e. (the most virulent two isolates (isolates No. 3 and 4) and the least virulent one (isolate No.5), the causal organism of cucumber wilt; on the susceptible cucumber genotype "Biet alpha" under greenhouse conditions. Data in Table (5) indicate that, all tested biological control agents minimized the disease symptoms parameters in comparing with the infected control treatments. *Trichoderma reesie* recorded the lowest pre-emergence damping-off against isolate No. 3 of *F. oxysporum* f.sp. *cucumerinum* (26.66%), while the highest pre-emergence was recorded by Th2 and *T. glaucum* against the same pathogenic isolate (46.66%). *Trichoderma (Th1), T. hamatum* and *T. glaucum* were completely controlled the post-emergence damping-off symptoms and gave 0.00%, while the rest bioagents *T. reesei, T. viride and T. harzianum 2* gave the highest post-emergence damping-off against the pathogenic isolate No 3; they recorded the same post-emergence value (6.66%). Disease severity index (DI) was at lowest value by *T. ressei* (40.00%), followed by *T . hamatum* (46.66%) while the highest DI value was recorded by Th2 (68.88%), followed by *T. viride* (62.22%) against the pathogenic isolate No. 3. The highest survival plants % was recorded by *T. ressei* (66.66%), while the lowest value was recorded by *T. glaucum* (19.99%), followed by *T. viride* (33.32%). This table indicate that all growth parameters of "Biet alpha" cucumber plants were improved by application of biological control agents against isolate No.3 of *Fusarium oxysporum* f.sp. *cucumerinum* comared with infested control treatments. The highest shoot fresh weight was recorded by *T. viride* (74.24 gm), followed by Th2 (72.88 gm), while the lowest shoot fresh weight was recorded by *T. glaucum* (32.48gm). Also, root fresh weight was at the highest value by *T. hamatum* (2.91 gm), followed by Th2 (2.86 gm), whereas the lowest root fresh weight was recorded by *T. glaucum* (1.08 gm). Taking into consideration data in Tables (5&6) it could also indicate that biological control agents great affected wilt incidence of cucumber plants "Biet alpha" infested by isolate No. 4 of the pathogen in comparing with control treatments. They minimized DI % and pre-& post-emergence damping-off, as well as they improved growth characters (shoot and root fresh weights). The lowest pre-emergence damping-off value was recorded in pots that treated with *T. reesei* (26.66%), followed by *T. viride* and *T. hamatum* (33.33%), while the highest pre-emergence value was recorded by Th1 (46.66%). Biological control agents well controlled disease incidence and minimized post-emergence damping-off to (0.00%) by all applied bioagents except *T. glaucum* (6.66%). *Trichoderma reesei* great affected disease incidence against isolate No. 4 and recorded the highest number of survival plants as well as the lowest DI% value (66.66 and 33.33%, respectively), followed by *T.hamatum* (43.33 and 66.66%, respectively). The lowest survival plants were recorded by *T. glaucum* (19.99%), followed by *T. viride* (26.66%), whereas the highest DI% was recorded in pots that treated with Th2 and *T. viride* (64.44 and 60.00%, respectively). Biological control agents improved vegetative growth characters in treated cucumber plants "Biet alpha". The highest shoot and root fresh weights were recorded by *T. reesei* (71.76 and 3.04 gm, respectively), while the lowest values were recorded by Th2 (35.18 and 1.21 gm, respectively). Regarding isolate No. 5 of the pathogen fungi; *Fusarium oxysporum* f.sp. *cucumerinum*; the same trend of isolate 4 results was noticed. The highest survival plants as well as the lowest DI% were recorded in plants that treated with *T. reesei* (80.00 and 20.00%, respectively) as compared with control treatments. The highest shoot and root fresh weight was recorded with *T. hamatum*, followed by *T. reesei* (86.16, 3.22, 78.22 and 3.02 gm, respectively). Sivakumar *et al.* (2000) reported that the effects of *T. harzianum* were due to both antibiosis and mycoparasitism. Mechanisms employed by *Trichoderma* species to effect biological control of plant diseases are many and complex, and their use varies with the kind of biocontrol agent, pathogen, and host plant involved in the interaction (Howell , 2003) Many investigators obtained similar results in greenhouse studies for controlling wilt disease on cucumber as well as other diseases (Ercole and Nipoti, 1986; Lifshitz *et al.*, 1986; Simochkina *et al.*, 1988; Khalifa, 1991; Ercole and Gennari, 1993 ; Lizuosen *et al.*, 2004; Zhu *et al.*, 2004; Rose *et al.*, 2004; Awad, 2004).

Table 5: Effect of some biological control agents on Fusarium wilt incidence of cucumber genotype "Beit alpha" incited by three isolates of *Fusarium oxysporum* f.sp. *cucumerinum* under greenhouse conditions.

Bioagent	Fungal isolate											
	3				4				5			
	Disease parameters %			Survival plants %	Disease parameters %			Survival plants %	Disease parameters %			Survival plants %
	Pre-Emergence	Post-Emergence	Disease index		Pre-Emergence	Post-Emergence	Disease index		Pre-Emergence	Post-Emergence	Disease index	
T. reesei	26.66	6.66	40.00	66.66	26.66	0.00	33.33	66.66	19.99	0.00	20.00	80.00
T. viride	39.99	6.66	62.22	33.32	33.33	0.00	60.00	26.66	46.66	0.00	60.00	39.99
T. harzianum (Th1)	39.99	0.00	50.00	59.99	46.66	0.00	53.33	46.66	26.66	0.00	36.66	66.66
T. hamatum	39.99	0.00	46.66	59.99	33.33	0.00	43.33	66.66	26.66	6.66	40.00	73.33
T. harzianum (Th2)	46.66	6.66	68.88	39.99	39.99	0.00	64.44	33.33	26.66	0.00	53.33	33.33
T. glaucum	46.66	0.00	57.55	19.99	39.99	6.66	55.55	19.99	26.66	6.66	48.88	46.66
Control	0.00	0.00	0.00	100.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	100.00
LSD at 5% among Isolates	7.69	3.76	10.75	10.00	7.69	3.76	10.75	10.00	7.69	3.76	10.75	10.00
LSD at 5% among Bioagents	5.02	4.58	9.89	4.06	5.02	4.58	9.89	4.06	5.02	4.58	9.89	4.06
LSD at 5% among Isolate X Bioagent	16.35	13.67	18.22	13.59	16.35	13.67	18.22	13.59	16.35	13.67	18.22	13.59

Table 6: Effect of biological control agents on growth *Characters* of cucumber genotype "Beit alpha" incited by three isolates of *Fusarium oxysporum* f.sp. *cucumerinum* under greenhouse conditions.

Bioagent	Fungal isolate								
	3			4			5		
	Disease index %	Growth parameters %		Disease index %	Growth parameters %		Disease index %	Growth parameters %	
		Shoot fresh weight (gm)	Root fresh weight (gm)		Shoot fresh weight (gm)	Root fresh weight (gm)		Shoot fresh weight (gm)	Root fresh weight (gm)
T. reesei	40.00	70.51	2.74	33.33	71.76	3.04	20.00	78.22	3.02
T. viride	62.22	74.24	2.43	60.00	54.72	1.73	60.00	60.52	2.22
T. harzianum (Th1)	50.00	63.04	2.61	53.33	64.28	2.58	36.66	66.72	2.59
T. hamatum	46.66	64.06	2.91	43.33	64.30	2.57	40.00	86.16	3.22
T. harzianum (Th2)	68.88	72.88	2.86	64.44	35.18	1.21	53.33	49.26	1.49
T. glaucum	57.55	32.48	1.08	55.55	50.54	1.68	48.88	57.66	2.10
Control	0.00	89.16	3.42	0.00	89.16	3.42	0.00	89.16	3.42
LSD at 5% among Isolates	10.75	12.97	0.49	10.75	12.97	0.49	10.75	12.97	0.49
LSD at 5% among Bioagents	9.89	14.61	0.51	9.89	14.61	0.51	9.89	14.61	0.51
LSD at 5% among Isolate X Bioagent	18.22	26.57	5.62	18.22	26.57	5.62	18.22	26.57	5.62

ACKNOWLEDGMENT

I would like to thank Prof. Dr. M.A. Awad , Professor of Plant Pathology, Faculty of Agriculture, Minufiya University, Egypt, who provided us with the pathogenic fungal isolates and the biological control agents.

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How to cite this article:

Majdah M.Y. Al-Tuwaijri. Studies on Fusarium wilt Disease of Cucumber. J App Pharm Sci, 2015; 5 (02): 110-119.