Molecular Studies on Pathological Variations of different isolates of *Fusarium oxysporum* f. sp.*niveum* the causal pathogen of watermelon wilt disease

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ABSTRACT

RAPD analysis was a useful tool for characterizing genetic variability among six isolates of *F. oxysporum* f. sp. *niveum* the causal pathogen of watermelon wilt disease including four virulent isolates, i.e. nos. 5, 24, 44, and 54 as well as two avirulent ones, i.e. nos. 8 and 51 which were previously identified by the authors according to their morphological and pathological characteristics. Genetic variability was observed among such isolates using five random primers. Out of five primers, three primers differentiate only some of the studied isolates but not all since similarity of 100% was recorded between isolates. However, primer 1 (AB1- 05) clearly distinguished between each of the all studied isolates, since no similarity of 100% was absolutely found among each of them. Interestingly, similar results were obtained when results of primers 1, 2 and 4 were statistically analyzed together into one dendrogram.

INTRODUCTION

Watermelon plants are exposed to many fungal diseases which affect quality and quantity of the crop. Watermelon wilt disease caused by *Fusarium oxysporum* f.sp. *niveum* (E.F. Sm.) Snyder & Hans., is a serious disease in many countries including Egypt (**Melchers**, 1931 and **Lin** *et al.*, 2009).

Differentiation of *Fusarium* spp. isolates by a polymerase chain reaction (PCR)-based method has been used recently to confirm the classification of *Fusarium* isolates based on pathogenicity tests. In recent years numerous molecular phylogeny markers that reveal the genetic diversity of similar organisms have arisen. Random amplified polymorphic DNA (RAPD) analysis is a fast, PCR-based method of genetic typing based on genomic polymorphisms (**Rahjoo** *et al.,* 2008,

Hashemi-Petroudil *et al.*, 2010, **Leong** *et al.*, **2010**.

The present work is an attempt .to study the possibility of using RAPD-PCR technique in differentiating between isolates of *Fusarium oxysporum* f.sp.*niveum* on the bases of their virulence .

MATERIALS AND METHODS

Molecular variation of six isolates of *F. oxysporum* f. sp. *niveum* using random amplified polymorphic DNA (RAPD-PCR) technique.

Six isolates of *F. oxysporum* f. sp. *niveum*, i.e. isolates nos. 5, 8, 24, 44, 51and 54 which were previously identified by the authors upon their morphological and pathological characteristics were used in the present study. These isolates were selected according to their virulence (**Elkazzaz** *et. al.*, 2012).

Identification of isolates of *F. oxysporum* f. sp. *niveum* using random amplified polymorphic DNA (RAPD-PCR) technique was carried out at Faculty of agriculture, Kafrelsheikh University.

Confirmation of Identification of this fungus was carried out using specific PCR primers.

Forward
5´-GTAAGCCGTCCTTCGCCTCG -3´
Reverse
5´-GCAAAATTCAATAGTATGGC -3´

Extraction and purification of DNA: The applied six isolates of such fungus were grown for 10 days at 28 ± 2°C in 250 ml flasks containing 100 ml Czapek's broth medium. Mycelium of each isolate was harvested by filtration, washed several times with sterile distilled water and blotted dry. Deoxyribonucleic acid (DNA)was isolated and purified from each isolate according to **Bowen** *et. al.* **(1996).**

Polymerase Chain Reaction (PCR) and Amplification Conditions: For random amplification polymorphic DNA (RAPD) analysis, the following 5-3, 10- mer random primers (Table 1) were used (supplied by Metabian Gmb H, Martinsried/ Germany).

Table (1): Catalog number and nucleotides sequence of the applied 5 decmer random primers.

Ser. No.	Cat. No.	Sequence
1	AB1- 05	5- CTGAGACGGA - 3.
2	AB1- 03	5- AGGACACTGC - 3.
3	AB1- 08	5- AAGGATCAGG - 3.
4	AB1-11	5- CAGGCCCTTC - 3.
5	AB1- 09	5- ACCACCTGGC - 3.

From the purified DNA 10µL containing 40 ng/ reaction were used as template to determine any molecular variation between the used six isolates of the fungus as 5 µL reaction of each random primer containing 12 ng applied to each reaction tube. The total volume was completed to 25µL using sterile distilled water. The amplification protocol was carried out according to **Qiagen (1997)**.

PCR product analysis: The amplified DNA was electrophorated using Hoefer HE 99x Max Submarine Electrophoresis unit on 2% agarose and IX TBE buffer at consistent 100 Volt for about three hr. The different bands sizes were determined against 100 bp ladder from Boechringer Mannheim. The separated bands were stained with ethidium bromide and documented using both Polaroid Instant camera and UV Transeliminator.

Phylogenetic tree construction:

The presence/absence matrix for amplified DNA fragments of the five ISJ markers was used to study the phylogenic relationships among the studied isolates. The statistical software NTSYS pc2.0 (**Rohlf, 2000**) was used to estimate the genetic relationships among the tested isolates. Employing the computer package NTSYS pc2.0, Nei and Lei's similarity coefficients (**Nei and Lei, 1979**) were calculated and used to establish genetic relationships among the isolates based on un-weighted pair group method of arithmetic means

(UPGMA) and sequential agglomerative hierarchical nested (SAHN) clustering

Statistics

Complete randomized design was applied to laboratory and greenhouses experiments. Data were tested by analysis of variance (**Little** and **Hills**, 1972).

RESULTS AND DISCUSSION

Six isolates of *F. oxysporum* f. sp. *niveum* the causal pathogen of watermelon wilt disease including four virulent isolates, i.e. nos. 5, 24, 44, and 54 as well as two avirulent ones, i.e. nos. 8 and 51 which were previously identified by the authors according to their morphological and pathological characteristics were used.

Relationship between six isolates of *F. oxysporum* f. sp. *niveum* using random Amplified Polymorphic DNA (RAPD) technique.

Identification of the six *F. oxysporum* f. sp. *niveum*, isolates was confirmed using specific primer (5′-GTAAGCCGTCCTTCGCCTCG -3′) (Fig.1). The results exacted that the six isolates were identified (100%) as *F. oxysporum*, since the 100 bp band of specific primer was detected in the genome.

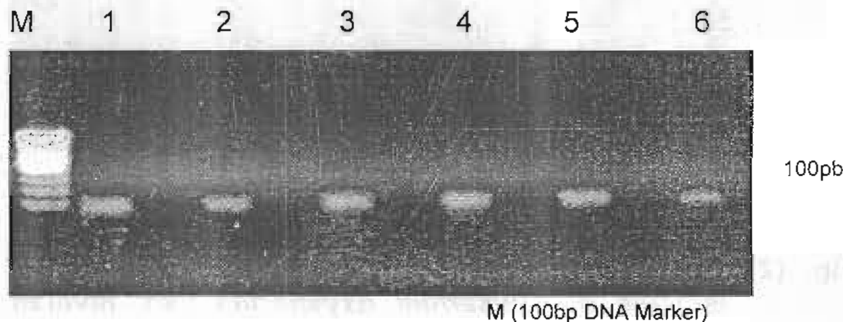

Fig.(1): Identification of six *Fusarium oxysporum* f. sp. *niveum* isolates by using specific primer (5 – GTAAGCCGTCCTTCGCCTCG-3) , M= molecular marker (100 bp ladder)

Data illustrated in figure (2) represent the genetic distances between the six studied *F. oxysporum* f. sp. *niveum* isolates according to RAPD-PCR by using primer AB 1-05. The phylognetic tree (Fig.3) divided these isolates into two main clusters, the first one includes isolates nos.54 and 51 which showed 84 % of similarity between each other and 50.0 % of similarity with isolate no.8 wich displayed the other two isolates . The second cluster was divided into three subclusters, since both isolates nos.5 and 24 showed 84 % of similarity. In the same time these two isolates were similar with isolates no.44 by 66.8% . The two main clusters exhibited similarity 38% between each other.

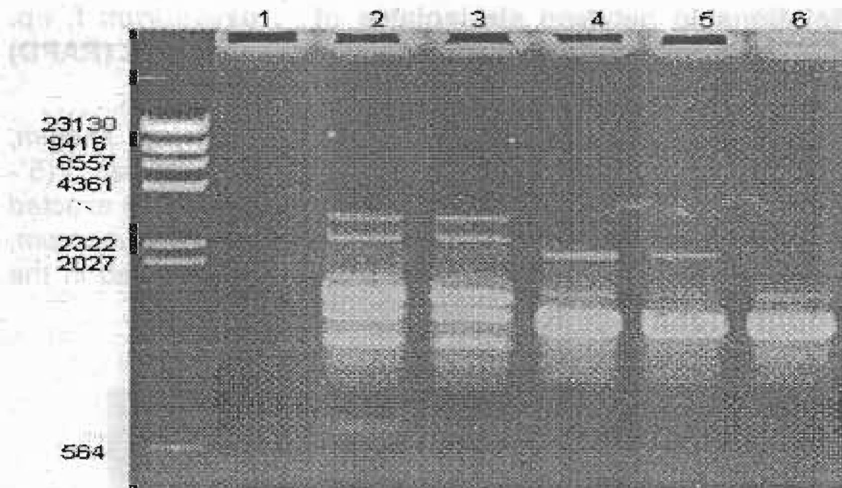

Fig. (2): Random Amplified DNA Polymorphisms of six isolates of *Fusarium oxysporum* f.sp. *niveum* with primer 1

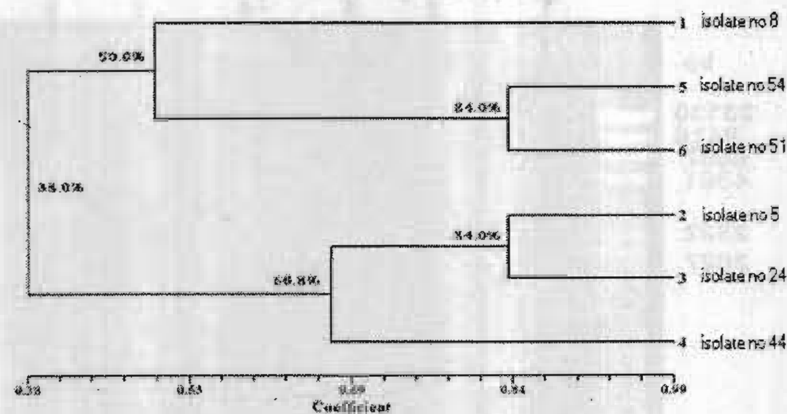

Fig. (3): Dendrogram based on RAPD-PCR products of six isolates of *Fusarium oxysporum* f.sp. *niveum* revealed by UPGMA cluster analysis of jaccard genetic similarity coefficients (primer 1) .

When primer AB 1-03 was used the six studied isolates of the fungus according to RAPD-PCR two groups were observed (Fig. 4). The phylognetic tree (Fig.5) divided the tested isolates into two clusters; the first one includes isolate nos.8 and 24 which showed 100% of similarity .These two isolates showed 74% similarity with isolate no.54. In the same time isolate no.51 showed 76% with the aforementioned three isolates. The second cluster was divided into two subclusters, since both isolates nos.5 and 44 showed 51 percent of similarity. The first cluster and the second one exhibited 25% similarity with each other.

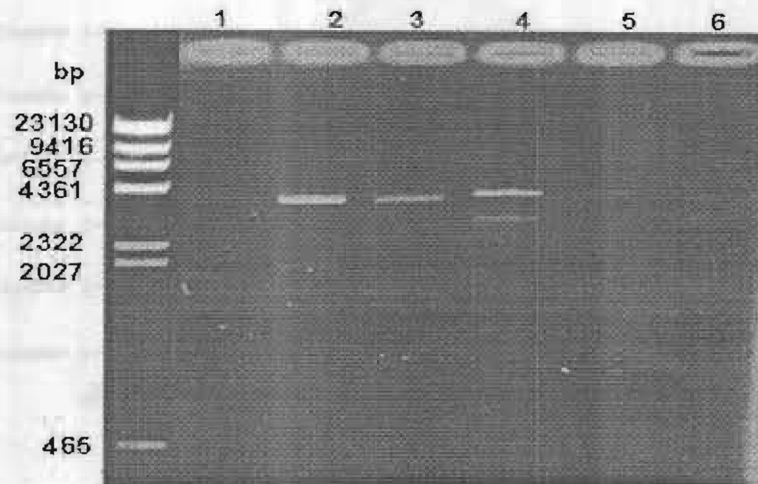

Fig. (4): Random Amplified DNA Polymorphisms of six isolates of *Fusarium oxysporum* f.sp. *niveum* with primer 2

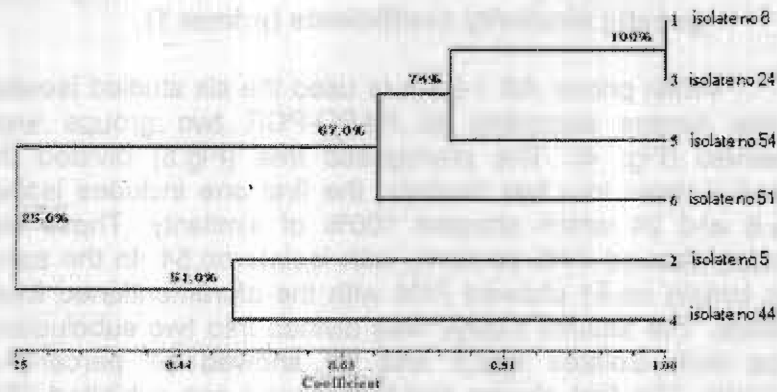

Fig. (5): Dendrogram based on RAPD-PCR products of six isolates of *Fusarium oxysporum* f.sp. *niveum* revealed by UPGMA sluster analysis of jaccard genetic similarity coefficients (primer 2) .

The genetic distances between the six studied isolates of such fungus by using primer AB 1-11 are shown in Fig (6). The phylognenetic tree (Fig.7) divided these tested isolates into two clusters, the first one includes isolates nos.8, 24, 5, 44 and

54 with 55.4% of similarity. The five isolates were shared 47% similarity to isolate no.51 .

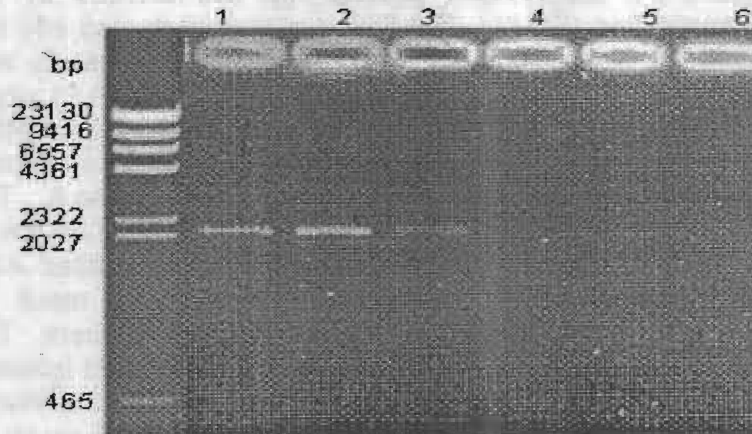

Fig. (6): Random Amplified DNA Polymorphisms of six isolates of *Fusarium oxysporum* f.sp. *niveum* with primer 4

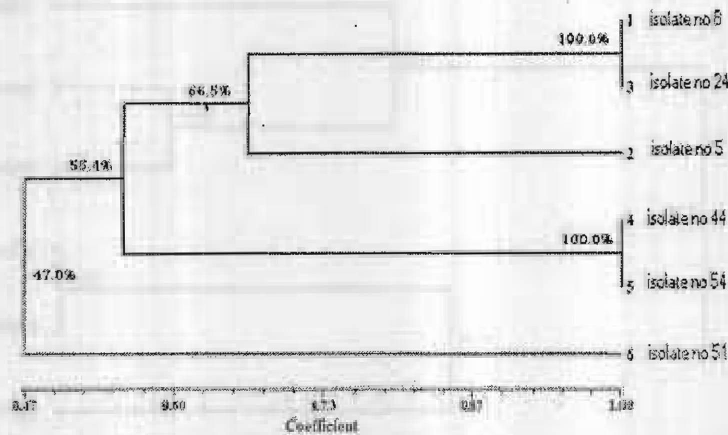

Fig. (7): Dendrogram based on RAPD-PCR products of six isolates of *Fusarium oxysporum* f.sp. *niveum* revealed by UPGMA sluster analysis of jaccard genetic similarity coefficients (primer 4) .

When results of primer 1 (AB 1-05), primer 2 (AB 1-03) and primer 4 (AB 1-11) were statistically analized together into

one dendogram no similarity of 100% was absolutely found among each of studied isolates. The six studied isolates of *F. oxysporum* f. sp. *niveum* divided into two clusters by the phylognetic tree (Fig.8). The first cluster was divided into two subclusters, since isolate no.5 showed 74% of similarity with isolate no.24 followed by isolate no.54 and isolate no.51 which exhibited 68.4% of similarity. The second cluster was divided into two subclusters, since isolate no.54 and isolate no.51showed 68.4% of similarity with isolate no.8. The subcluster of isolate no.5, and isolate no.24 showed 54.8% of similarity with isolate no.44. The dendrogram exhibited 43% similarity between all *Fusarium* isolates as a result of application of three different decmer random primers. The phylognetic tree differentiated between the four tested isolates, i.e. isolate nos.8 , 44 , 51 and 54 since the 100 % of similarity among these four isolates was not detected . But it could be differentiated between isolate no.5 and no.24, since the similarity of 74.0 % was detected.

Results of AB1- 08 (5- AAGGATCAGG - 3) and AB1- 09 (5-ACCACCTGGC - 3) were insignificant.

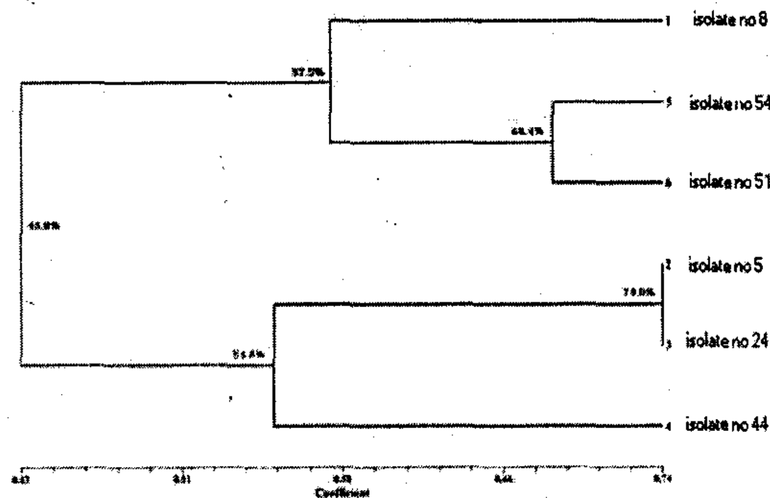

Fig. (8): Dendrogram showing Polymorphisms of DNA six isolates of *Fusarium oxysporum* f.sp. *niveum* revealed by UPGMA cluster analysis of jaccard genetic similarity coefficients calculated from RAPD (primer 1,2,4) .

These results were supported by **Turner** *et al.*, 1998 who stated that the primers were able to differentiate between *F. avenaceum* and *F. tricinctum*. Also **Vakalounakis** and **Fragkiadakis** (1999) confirmed our results by differentiating between 106 isolates of *F. oxysporum*, using RAPD analysis. They reported that RAPD analysis was effective in distinguishing isolates of *F. oxysporum* f. sp. *radicis-cucumerinum* from those of *F. oxysporum* f. sp .*cucumerinum*. Similary, **Mostafa** ***et al.***, 2002 and **Ahmed** *et al.*, 2009 reported that RAPD analysis was utilized to discriminate between highly virulent (HV) and weakly virulent (WV) isolates of *F. oxysporum* in Iran and between isolates of *F. oxysporum* f sp . *lycopercisi* in Egypt, respectivelly.

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الملخص العربي

دراسات البيولوجيا الجزيئية على الاختلافات المرضية لعزلات مختلفة من الفطر فيوزاريوم اوكسيسبوريوم شكل نيفم المسبب لمرض ذبول البطيخ

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يعتبر تحليل ال **RAPD** (التضاعف العشوائى لمناطق متباينة من الحمض النووى **DNA**) من التقنيات المفيدة والتى استخدمت فى هذة الدراسة فى وصف الاختلافات الوراثية لستة عزلات من الفطر فيوزاريوم اوكسيسبورم شكل نيفم (***Fusarium oxysporum* f. sp. *niveum***) المسبب لمرض ذبول البطيخ . وكانت هذة العزلات المرضية عبارة عن العزلات رقم 5 ، 24 ، 44 ، 55 بالاضافة الى عزلتين غير ممرضتين من نفس المسبب المرضى السابق ذكرة رقمى 25 ، 28 وقد تم تعريف هذة العزلات مسبقا طبقا للخصائص المورفولوجية والمرضية. استخدم فى هذة الدراسة خمسة بادئات عشوائية للحمض النووى **DNA** وقد تم ملاحظة وجود اختلافات وراثية بين تلك العزلات باستخدام هذة الخمسة بادئات. وقد أظهرت ثلاث بادئات من تلك الخمسة وجود اختلافات بين بعض تلك العزلات تحت الدراسة حيث أن البادئات الأخرى أظهرا وجود قرابة بنسبة 100% بين بعض تلك العزلات . بصفة عامة فان البادئ رقم 1 (**AB1-05**) قد فرق بين جميع العزلات تحت الدراسة حيث لم يظهر قرابة 100% بين أى من العزلات والأخرى والجدير بالذكر بالذكر أن نفس النتيجة السابقة تم الحصول عليها عندما تم تحليل النتائج المتحصل عليها من استخدام البادئات 1 ، 2 ، 4 معا فى صورة شجرة قرابة.