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DETECTION OF GENETIC VARIATION IN SOME EGYPTIAN COTTONS RESISTANCE OR SUSCEPTIBLE TO FUSARIUM WILT DISEASE BY RAPD ANALYSIS

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ABSTRACT

Random amplified polymorphic DNA (RAPD) analysis was used to evaluate the genetic diversity of 5 experimental crosses and 9 commercial cultivars of Egyptian cottons (*Gossypium barbadense* L.). The crosses showed varying levels of susceptibility to Fusarium wilt disease, while all the cultivars were highly resistant. The tested genotypes (crosses and cultivars) were analyzed with 4 random decamer primers using the polymerase chain reaction (PCR). All the primers detected polymorphism in all the tested genotypes. Cluster analysis by the unweighted pair-group method of arithmetic means (UPGMA) placed the genotypes in several groups with overall similarity levels ranged from 67.15 to 96.74%, which may indicate that the tested genotypes had a narrow genetic base. Grouping the genotypes based on their RAPD-PCR banding patterns was not related to their reaction to the Fusarium-wilt disease. The results of this study could be used for cultivar identification or for seed purity tests.

INTRODUCTION

Fusarium wilt (*Fusarium oxysporum* Schlect. f.sp. *vasinfectum* (Atk.) Snyd. and Hans) of cotton (*Gossypium* spp.) has long been known in the Nile Valley, where it caused serious losses in the commercial Egyptian cottons (*G. barbadense* L.) in the late fifties (Bakry *et al.*, 1958). Since then, an extensive cotton-breeding program was initiated to develop cultivars resistant to the disease. In this program, cotton genotypes are screened under greenhouse conditions, in soil infested with the wilt fungus.

Currently, screening of breeding materials under greenhouse conditions is the only reliable method to distinguish the Fusarium-wilt highly resistant genotypes. The test is time consuming, and may be influenced by variability inherent in the experimental system (A.A. Aly, *personal observations*). The first symptoms of the disease appear on susceptible genotypes after 20 days from planting date under very favorable environmental conditions and may require a longer period of time under less favorable conditions.

Therefore, another reliable method, either alternative or complementary to greenhouse tests, is required for identification of the Fusarium-wilt highly resistant genotypes.

Genetic diversity among genotypes of plants can be evaluated with seed proteins, isozymes, and DNA markers (Gepts, 1993). However, a large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner. This limits the use of isozymes, which are few or lack adequate level of polymorphism in *Gossypium* (Tatineni *et al.*, 1996). Molecular genetic markers have developed into powerful tools to analyze genetic relationships and genetic diversity. Restriction fragment length polymorphisms (RFLPs) can be used, but they

are costly and time-consuming to evaluate. Random amplified polymorphic DNA (RAPD) is a useful technique to evaluate taxonomic identity and kinship (Hadrys *et al.*, 1992). RAPDs were shown to provide a level of resolution equivalent to RFLPs for determining genetic relationships among *Brassica oleracea* L. genotypes (Dos Santos *et al.*, 1994) and among *B. napus* L. breeding lines (Hallden *et al.*, 1994). The technical simplicity and speed of RAPD methodology is a principal advantage (Gepts, 1993). Estimates of similarity based on RAPDs have been developed for *Gossypium* (Multani and Lyon, 1995; Tatineni *et al.*, 1996; Iqbal *et al.*, 1997; Vroh *et al.*, 1997; Wajahatullah and Stewart, 1997; Khan *et al.*, 2000 and Pendse *et al.*, 2001).

The present investigation was initiated to determine whether the Fusarium-wilt resistant genotypes of cotton can be distinguished by using RAPD analysis.

MATERIALS AND METHODS

Evaluation of cotton genotypes for Fusarium wilt-resistance or susceptibility:

The genotypes evaluated in this test were submitted by Cotton Breeding Section, Cotton Research Institute.

The inoculum used in the test was a mixture of equal parts (w/w) of 50 isolates of *F. oxysporum* f.sp. *vasinfection* (FOV) race 3. These isolates were obtained from the fungal collection of Cotton Pathology Lab., Plant Path. Res. Inst., Agric. Res. Cent., Giza. Autoclaved clay loam soil was infested with the mixture of isolates at a rate of 10 g/kg of soil. Substrate for growth of each isolate was prepared in 500 ml glass bottles, each bottle contained 50 g of sorghum grains and 40 ml of tap water. Contents of bottle were autoclaved for 30 minutes. Isolate inoculum, taken from one-week old culture on PDA, was aseptically introduced into the bottle and allowed to colonize sorghum for 3 weeks. Infested soil was dispensed in 25-cm diameter clay pots and these were planted with 20 seeds per pot. There were 5 replications (pots) for each genotype.

Pots were distributed on a greenhouse bench in a randomized complete block design of 5 replications. The greenhouse was equipped with a heating system assuring that the minimum temperature in the greenhouse was maintained at 28°C; however, due to the lack of a cooling system, the maximum temperature was out of control fluctuating from 30 to 35°C depending on the prevailing temperature during the day (the test was conducted in January and February, 2002). Percentage of wilted seedlings were recorded 40 days from planting date.

Statistical analysis of the greenhouse test:

Analysis of variance (ANOVA) of the data was performed with the MSTAT-C statistical package (A Microcomputer Program for the Design, Management, and Analysis of Agronomic Research Experiments, Michigan State Univ., USA). Duncan's multiple range test was used to compare genotypes means. Percentage data were transformed into arc sine angles before carrying out ANOVA to produce approximately constant variance.

DNA isolation

DNA was isolated from 50 mg of each genotype by using Qiagen kit for DNA extraction. The extracted DNA was dissolved in 100 µl of the elution buffer. The concentration and purity of the obtained DNA was determined by using “Gen qunta” system-Pharmacia Biotec. The purity of the DNA for all samples in this study ranged between 90-97% and with ratio between 1.7-1.8. Concentration was adjusted to give 6 ng/µl for all samples by using TE buffer, pH 8.0.

Random amplified polymorphism DNA (RAPD) technique

Thirty ng from the extracted DNA were used for amplification reaction. The polymerase chain reaction (PCR) mixture contained PCR beads tablet (Amessham Pharmacia Biotec.), containing all the necessary reagents except the primer and the DNA.

The Amessham Pharmacia Biotec kits contain 4 primers were used. The sequences of the primers were as follows:

RAPD Analysis Primer 1: d (CGTGCGGGAA)-3
RAPD Analysis Primer 2: d (GTTTCGCTCC)-3
RAPD Analysis Primer 5: d (AACGCGCAAC)-3
RAPD Analysis Primer 6: d (CCCGTCAGCA)-3

Five microliters of the primer (10 mer) were added. The total volume was completed to 25 µl by using sterile distilled water. The amplification protocol was carried out as follows using PCR unit II biometra.

a) Denaturation at 95°C for 5 minutes.
b) 45 cycles each consists of the following steps:
   1. Denaturation at 95°C for a minute.
   2. Annealing at 36°C for a minute.
   3. Extension at 72°C for 2 minutes.
c) Final extension at 72°C for 5 minutes.
d) Hold at 4°C.

Seven µl of 6X tracking buffer (manufactured by Qiagen kit) were added to 25 µl of the amplification product.

Amplification product analysis

Two methods were used for electrophoresis technique:

1. The amplified DNA for all samples (15 µl) were electrophoresed using electrophoresis unit (WIDE mini-sub-cell GT Bio-RAD) on 2% agarose containing ethedium bromide (0.5 µg/ml). At 75 constant volt, and determined with UV transilluminator.
2. The PCR products were separated and determined by using polyacrylamide gel electrophoresis slabs according to Pieter *et al.* (1995). The gel matrix consisted of the following components:

<u>TBE 10x pH 8.3</u>

Boric acid	11.0 g
Tris base	21.6 g
EDTA anhydrouse	1.168 g
D.D. water	200 ml
TBE 10 x pH 8.3	4.0 ml
Acrylamide 40%	4.7 ml

Bisacrylamide 2%	3.0 ml
10% (w/v) APS	300 μl
D.D. Water	28 ml
Temed	20 μl

Eight μl from each sample were added to well of electrophoresis unit (Hoefer SE 600 series Pharmacia). The running was done at 2 mA per sample. The running took about 1.5 hours. Gels were stained with silver nitrate (Bassam *et al*., 1991).

Gel Analysis

A gel documentation system was used (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6C, Fullerton CA 92631). The similarity levels were determined by the unweighted pair-group method based on arithmetic mean (UPGMA) and Pearson's correlation coefficient.

RESULTS AND DISCUSSION

Resistance or susceptibility of the genotypes to Fusarium wilt is shown in Table 1. The genotypes were classified into two distinct groups. The susceptible group included all the experimental crosses, while the resistant group included all the commercial cultivars. Of the susceptible genotypes, family 478/98 showed the highest level of susceptibility, while family 19/99 showed the lowest level of susceptibility. The other genotypes showed intermediate levels of susceptibility between these two extremes.

Table 1. Evaluation of cotton genotypes for Fusarium-wilt resistance or susceptibility under greenhouse conditions.

Genotype	Family	Wilted seedlings[a] (%)[b]
Cross Giza 85 x Australian cultivar	478/98	100.00[c] A
Cross Giza 80 x Australian cultivar	28/99	34.34 D
Cross Giza 83 x Australian cultivar	45/99	57.86 C
Cross Giza 80 x Australian cultivar	19/99	16.80 D
Cross Giza 80 x Australian cultivar	545/98	73.22 B
Cultivar Giza 89		0.00 E
Cultivar Giza 45		0.00 E
Cultivar Giza 86		0.00 E
Cultivar Giza 88		0.00 E
Cultivar Giza 70		0.00 E
Cultivar Giza 85		0.00 E
Cultivar Giza 80		0.00 E
Cultivar Giza 83		0.00 E
Cultivar Giza 87		0.00 E

[a] Wilted seedlings were calculated according to the following formula: [Wilted seedlings/emerging seedlings]x100.

[b] Percentage data were transformed into arc sine angles before carrying out the analysis of variance to produce approximately constant variance.

[c] Means followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test. Each value is the mean of five replicates (pots).

In this study, the genotypes were screened in the greenhouse under conditions very favorable for unrestricted development of the wilt fungus. The soil was sterile, temperature was optimal most of the time, and the inoculum density was relatively high. However, the wilt fungus FOV was unable to infect any of the commercial cultivars.

Figs.1B-4B showed the phenograms constructed based on the similarity levels (SLs) generated from cluster analysis of RAPD banding patterns (Figs. 1A-4A), generated by using primers nos. 1, 2, 5 and 6, respectively. The greater the SL the more closely the genotypes were. Primers nos. 1 and 2 detected reasonable levels of polymorphism among the tested genotypes. Hence, the overall SLs generated by these primers were 83.81 and 67.15%, respectively (Figs. 1B and 2B). On the other hand, primers nos. 5 and 6 produces a very low level of polymorphism among the genotypes. Therefore, the SLs were 95.69 and 96.74%, respectively (Figs. 3B and 4B). These results led us to conclude that primers nos. 5 and 6 were not appropriate to detect the genetic variation among the genotypes. These results also indicate that the tested genotypes had a narrow genetic base. Therefore, the present discussion will consider only the phenograms shown in Figs. 1B and 2B.

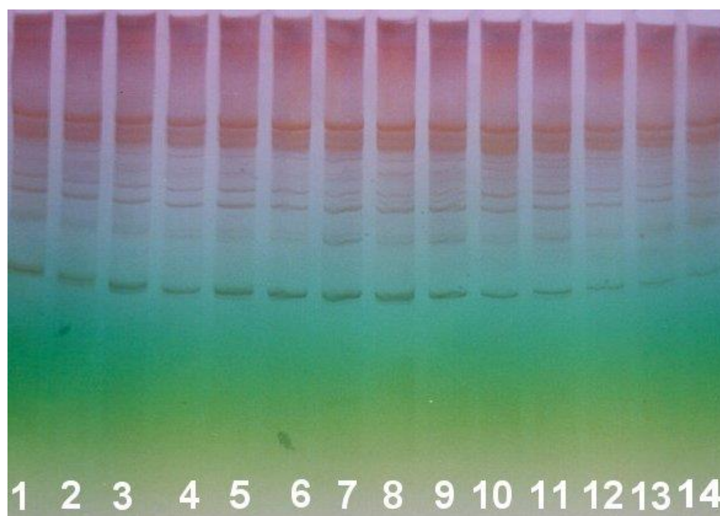

Fig. (1A): RAPD banding patterns of cotton genotypes obtained by the primer no. 1 and electrophoresed on polyacrylamide gel. Lanes from left to right were Family 478/98 (1), Family 28/99 (2), Family 45/99 (3), Family 19/99 (4), Family 545/98 (5), Cultivar Giza 89 (6), Cultivar Giza 45 (7), Cultivar Giza 86 (8), Cultivar Giza 88 (9), Cultivar Giza 70 (10), Cultivar Giza 85 (11), Cultivar Giza 80 (12), Cultivar Giza 83 (13), and Cultivar Giza 87 (14).

Primer no.1 placed the genotypes in three distinct subclusters at SLs 88.58, 93.54 and 94.66% (Fig. 1). Since each subcluster included both resistant and susceptible genotypes, it was concluded that grouping the

genotypes by using this primer was not related to their reaction to Fusarium wilt- that is, it was not possible to differentiate the Fusarium-wilt resistant genotypes by using this primers. On the other hand, this primer was able to differentiate between some of the commercial cultivars, which were placed in remotely related subclusters. For example, this primer showed a distant affinity between Giza 80 and Giza 70. This cluster also distinguished Giza 70 from any of the cultivars Giza 88, Giza 89, Giza 45 and Giza 86.

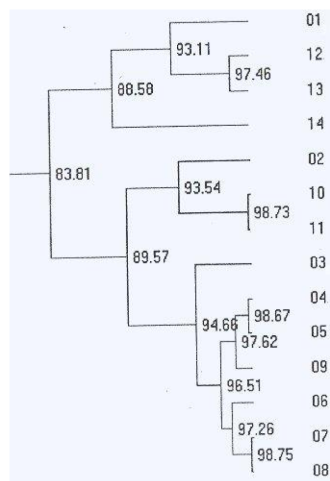

Fig. (1B): Phenogram based on cluster analysis of RAPD banding patterns of cotton genotypes obtained by the primer no. 1 and electrophoresed on polyacrylamide gel. Lanes from left to right ere Family 478/98 (1), Family 28/99 (2), Family 45/99 (3), Family 19/99 (4), Family 545/98 (5), Cultivar Giza 89 (6), Cultivar Giza 45 (7), Cultivar Giza 86 (8), Cultivar Giza 88 (9), Cultivar Giza 70 (10), Cultivar Giza 85 (11), Cultivar Giza 80 (12), Cultivar Giza 83 (13), and Cultivar Giza 87 (14).

Primer no. 2 produced the highest level of polymorphism among the genotypes (Fig. 2). Hence, the overall SL was reduced to 67.16% by using this primer. Certain details in Fig. (2B) are worthy of mention. Most of the resistant genotypes were placed in subclusters remotely related to those, which included the susceptible genotypes. However, the resistant cultivar Giza 89 was a notable exception because it was included in the same subcluster with the susceptible families 45/99, 19/99 and 545/98 (SL = 92.43%). Each of Giza 45, Giza 87, and Giza 70 showed a distant affinity from the other commercial cultivars. The results of this study indicate that primers nos. 1 and 2 could be of practical value for cultivar identification or for seed purity tests, especially if one take into account that different commercial cultivars may be grown in adjacent locations in Egypt. In fact, this situation is frequently encountered in cotton production areas particularly in the Nile Delta.

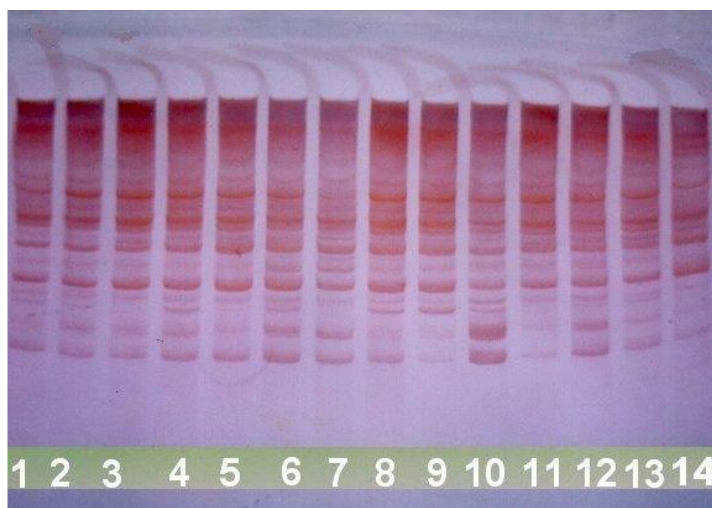

Fig. (2B): **RAPD banding patterns of cotton genotypes obtained by the primer no. 2 and electrophoresed on polyacrylamide gel. Lanes from left to right were Family 478/98 (1), Family 28/99 (2), Family 45/99 (3), Family 19/99 (4), Family 545/98 (5), Cultivar Giza 89 (6), Cultivar Giza 45 (7), Cultivar Giza 86 (8), Cultivar Giza 88 (9), Cultivar Giza 70 (10), Cultivar Giza 85 (11), Cultivar Giza 80 (12), Cultivar Giza 83 (13), and Cultivar Giza 87 (14).**

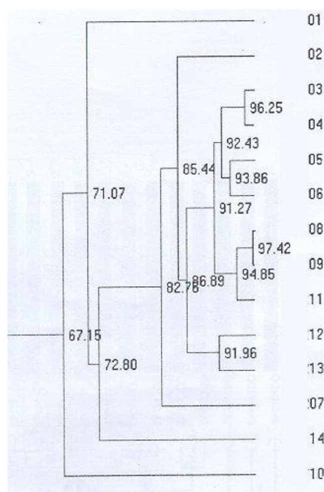

Fig. (2B): Phenogram based on cluster analysis of RAPD banding patterns of cotton genotypes obtained by the primer no. 2 and electrophoresed on polyacrylamide gel. Lanes from left to right were Family 478/98 (1), Family 28/99 (2), Family 45/99 (3), Family 19/99 (4), Family 545/98 (5), Cultivar Giza 89 (6), Cultivar Giza 45 (7), Cultivar Giza 86 (8), Cultivar Giza 88 (9), Cultivar Giza 70 (10), Cultivar Giza 85 (11), Cultivar Giza 80 (12), Cultivar Giza 83 (13), and Cultivar Giza 87 (14).

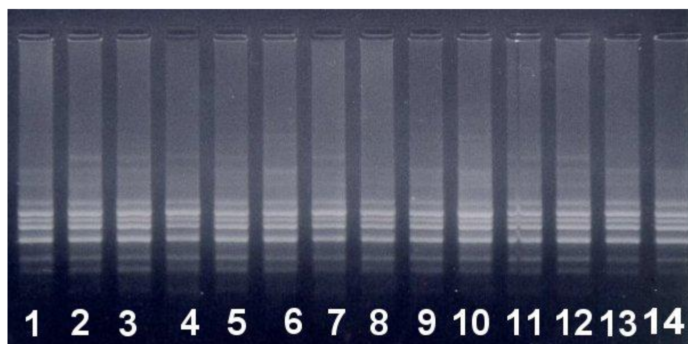

Fig. (3A): **RAPD banding patterns of cotton genotypes obtained by the primer no. 5 and electrophoresed on agaros gel. Lanes from left to right were Family 478/98 (1), Family 28/99 (2), Family 45/99 (3), Family 19/99 (4), Family 545/98 (5), Cultivar Giza 89 (6), Cultivar Giza 45 (7), Cultivar Giza 86 (8), Cultivar Giza 88 (9), Cultivar Giza 70 (10), Cultivar Giza 85 (11), Cultivar Giza 80 (12), Cultivar Giza 83 (13), and Cultivar Giza 87 (14).**

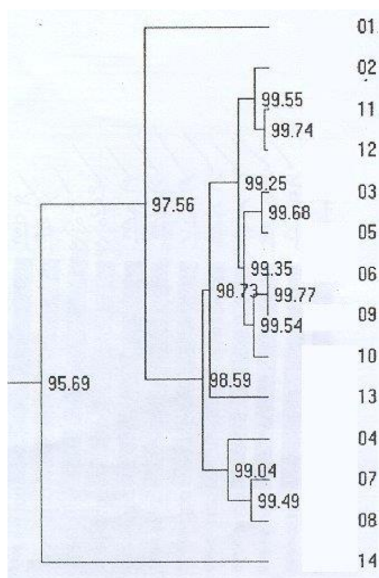

Fig. (3B): **Phenogram based on cluster analysis of RAPD banding patterns of cotton genotypes obtained by the primer no. 5 and electrophoresed on agaros gel. Lanes from left to right were Family 478/98 (1), Family 28/99 (2), Family 45/99 (3), Family 19/99 (4), Family 545/98 (5), Cultivar Giza 89 (6), Cultivar Giza 45 (7), Cultivar Giza 86 (8), Cultivar Giza 88 (9), Cultivar Giza 70 (10), Cultivar Giza 85 (11), Cultivar Giza 80 (12), Cultivar Giza 83 (13), and Cultivar Giza 87 (14).**

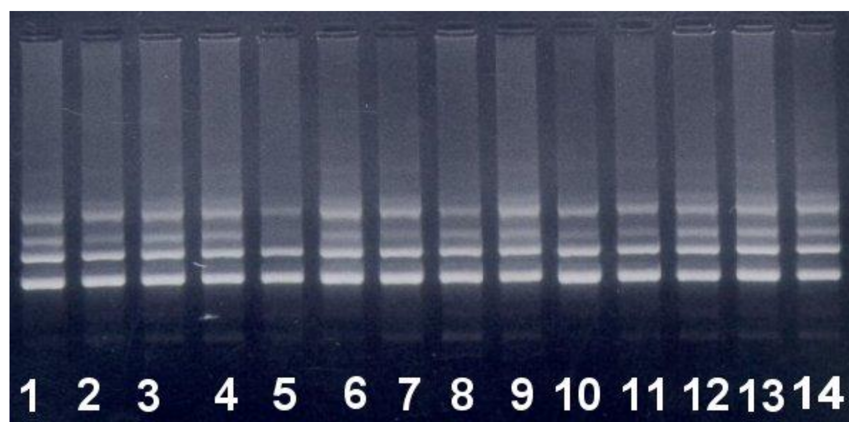

Fig. (4A): RAPD banding patterns of cotton genotypes obtained by the primer no. 6 and electrophoresed on agarose gel. Lanes from left to right were Family 478/98 (1), Family 28/99 (2), Family 45/99 (3), Family 19/99 (4), Family 545/98 (5), Cultivar Giza 89 (6), Cultivar Giza 45 (7), Cultivar Giza 86 (8), Cultivar Giza 88 (9), Cultivar Giza 70 (10), Cultivar Giza 85 (11), Cultivar Giza 80 (12), Cultivar Giza 83 (13), and Cultivar Giza 87 (14).

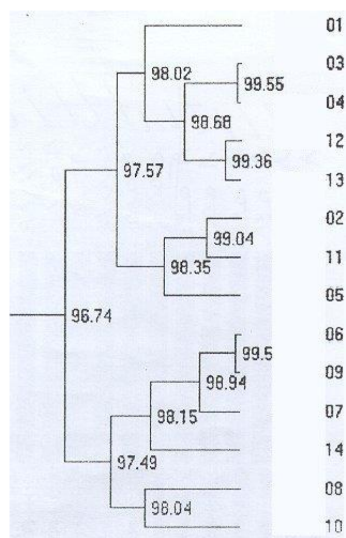

Fig. (4): Phenogram based on cluster analysis of RAPD banding patterns of cotton genotypes obtained by the primer no. 6 and electrophoresed on agarose gel. Lanes from left to right were Family 478/98 (1), Family 28/99 (2), Family 45/99 (3), Family 19/99 (4), Family 545/98 (5), Cultivar Giza 89 (6), Cultivar Giza 45 (7), Cultivar Giza 86 (8), Cultivar Giza 88 (9), Cultivar Giza 70 (10), Cultivar Giza 85 (11), Cultivar Giza 80 (12), Cultivar Giza 83 (13), and Cultivar Giza 87 (14).

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الكشف عن التباين الوراثى فى بعض الأقطان المصرية المقاومة أو القابلة للإصابة بمرض ذبول الفيوزاريوم باستعمال تقنية التضاعف العشوائى لمناطق متباينة من الحمض النووى دى. إن. إيه.

عزت محمد حسين ، ماجى السيد محمد ، محمد سيد خليل، على عبد الهادى على
معهد بحوث امراض النباتات - مركز البحوث الزراعية - الجيزة - مصر.

استعملت تقنية التضاعف العشوائى لمناطق متباينة من الحمض النووى دى. إن. إيه لتقييم درجة التنوع الوراثى فى خمسة هجن تجريبية وتسعة أصناف تجارية من الأقطان المصرية. تباينت الهجن فيما بينها من حيث درجة القابلية للإصابة بمرض ذبول الفيوزاريوم، أما الأصناف التجارية فكانت كلها على درجة عالية من المقاومة للمرض. إستعملت أربعة بادئات عشوائية لإجراء تفعل البوليميريز المتسلسل على الحمض النووى المستخلص من التراكيب الوراثية موضع الدراسة (الهجن والأصناف). تمكنت جميع البادئات من إحداث تضاعف للحمض النووى. إستعمل التحليل العنقودى لتصنيف هذه التراكيب الوراثية إلى محموعات، بناء على مابينها من تماثل فى أنماط الحمض النووى، وتم التعبير عن النتائج فى فينوجرامات. أظهرت الدراسة أن درجة التماثل الكلى بين أنماط الحمض النووى للتراكيب الوراثية تراوحت من ٦٧,١٥ إلى ٩٦,٧٤%، حسب نوع البادئ المستخدم، مما يدل على أن التراكيب موضع الدراسة متقاربة من الناحية الوراثية. إن تقسيم التراكيب الوراثية إلى مجموعات بناء على درجة التماثل فى الحمض النووى لم يكن مرتبطاً بدرجة قابلية التراكيب الوراثية للإصابة بمرض ذبول الفيوزاريوم. تدل نتائج الدراسة الحالية على أنه من الممكن إستعمال أنماط الحمض النووى المميزة للتراكيب الوراثية، والمتحصل عليها باستعمال تقنية التضاعف العشوائى، كوسيلة جزيئية لتمييز أصناف القطن التجارية، أو لإجراء إختبارات النقاوة للتقاوى.