USING RAPD-PCR TECHNIQUES IN IDENTIFICATION OF COTTON FUSARIA

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(Manuscript received 26 September 2004)

Abstract

The development of wilt symptoms is the main criterion for the identification of such isolates *F. oxysporum* f. sp. *vasinfectum*. This biological test is time consuming and sensitive to temperature changes. Therefore, differentation between *F. oxysporum* f. sp. *vasinfectum* isolates from the recently isolated damping-off producing isolates of *F. oxysporum* are being explored by RAPD-PCR using any of the primers d (CGTGCGGGAA)-3 or d (AAGAGCCCGT)-3 or d (AACGCGCAAC)-3 or d (CCCGTCAGCA)-3 by RAPD-PCR. The method was found sensitive, accurate, practical and time saving in recognizing *F. oxysporum* f. sp. *vasinfectum* isolates, which showed a very high degree of similarity. In contrast, the damping-off causing *F. oxysporum* isolates tested showed different clusters with a low overall similarity.

Ker words: *F. oxysporum* f. sp. *vasinfectum*, Fusarium wilt, *F. oxysporum* damping-off, cotton, RAPD-PCR.

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). The fungus is endemic in the soil, and the fungicides are not effective *in vivo*, thus an extensive cotton-breeding program had long been initiated to develop resistant cultivars to the disease. In such a program, cotton genotypes are screened under greenhouse conditions, in soil artificially infested with virulent isolates of the wilt fungus (Colyer, 1988).

Screening of breeding materials under greenhouse conditions is practically the only reliable method to select the resistant genotypes as well as identifying the wilt inducing Fusaria using susceptible cultivars. The test is time consuming, and may be influenced by the variability inherent in the experimental system (Aly, *personal observations*). The disease onset is recognized on susceptible genotypes after 20 days from planting under favorable conditions and may require a longer period under less favorable conditions. Aly *et al.* (1996) has recently reported a damping-off inducing, non-wilt producing isolates of *F. oxysporum* identified on the basis of cultural characteristics and pathogenic behavior on wilt-susceptible cotton.

The use of various nucleic acid-based techniques for the detection and identification of microorganisms has dramatically increased in recent years and may provide highly specific tools for such purposes. Restriction fragment length polymorphisms (RFLPs), which is costly and time consuming can be used; however, the random amplified polymorphic DNA (RAPD) is also a useful technique to evaluate taxonomic identity. The application of (RAPD) to produce isolate-specific DNA-fingerprints is especially promising (Welsh and McClelland, 1990 and Williams *et al.*, 1990). This technique has the advantage of that no DNA base sequence information of the organism is needed. The technical simplicity and speed of RAPD methodology is a principal advantage over other techniques (Gepts, 1993).

The objective of the present work was to explore the possible utilization and efficiency of some DNA-based techniqués in differentiating the wilt isolates *F. oxysporum* f.sp. *vasinfectum* from other *F. oxysporum* producing damping-off, as a rapid and reproducible alternative and/or a complementary method to the greenhouse test.

MATERIALS AND METHODS

Identification and differentiation of seedling wilt and damping off Fusaria:

The isolates of *F. oxysporum* (Fo) and *F. oxysporum* f. sp. *vasinfectum* (Fov) used in this work were obtained from the fungal collection of the Department of Cotton Diseases, Plant Path. Res. Inst., Agric. Res. Center, Giza (Table 1). Isolates (1 to 8) are *Fusarium oxysporum*, based on cultural characters; however, causing seedling damping-off with severity ranging from 45 % to 67.5 % on Giza 80 variety with no wilt symptoms on Giza 74, while the isolates (9 to 14) are *F. oxysporum* f. sp. *vasinfectum* causing wilt symptoms with severity ranging from 40 % to 67.5 % on Giza 74 variety in greenhouse tests.

Table 1. Original source, serial number and the percentage of the resulting disease infection.

Original source and/or Collection Designation	Serial number	% of Infection
Cotton 76	1	65 % damping-off
Cotton	2	67.5 % damping-off
Cotton 64	3	60 % damping-off
Cotton 66	4	50 % damping-off
Cotton 27	5	52.5 % damping-off
Cotton 71	6	57.5 % damping-off
Cotton 2	7	45 % damping-off
Cotton 10	8	52.5 % damping-off
F.V. 1	9	60 % wilt
F.V. 5	10	40 % wilt
F.V. 4	11	42.5 % wilt
F.V. 2	12	52.5 % wilt
F.V. 7	13	60 % wilt
F.V. 3	14	67.5 % wilt
F.V. 6	15	59 % wilt

DNA isolation:

DNA was isolated from 50 mg mycelium of each isolate grown on liquid PD medium for one week 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 and 97 % with ratio of 1.7 - 1.8. Concentration was adjusted to 6 ng/µl for all samples using TE buffer (tris-HCl and EDTA), pH 8.0.

Random amplified polymorphism DNA (RAPD) technique:

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

The base sequences of the four primers used were as follows:

	(G + C %)
Primer 1: d (CGTGCGGGAA)-3	70 %
Primer 4: d (AAGAGCCCGT)-3	60 %
Primer 5: d (AACGCGCAAC)-3	50 %
Primer 6: d (CCCGTCAGCA)-3	40 %

Five microliters of any given primer (10 mer) were added to the mixture containing the fungal DNA. The total volume was completed to 25 µl with sterile distilled water. The amplification protocol using PCR unit II biometra was carried out as follows:

- Denaturation at 95°C for 5 minutes and 45 cycles each consists of the following steps;
- Denaturation at 95°C for 1 minute, annealing at 36°C for 1 minute, extension at 72°C for 2 minutes. Final extension at 72°C for 5 minutes and 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:

The amplified DNA for all samples (15 µl) were electrophoresed using WIDE mini-sub-cell GT Bio-RAD unit on 2% agarose containing ethedium bromide (0.5 µg/ml) at a constant 75 volt power for 90 minutes and 60 mA. Visualization was made with UV transilluminator. AAB documentation system (Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6 C, Fullerton CA 92631) was used to draw the levels of similarity. The different molecular weights of bands were determined against a DNA standard (G316A Promega Inc., USA) with molecular weights 1000, 750, 500, 150, and 50 bp.

RESULTS and DISCUSSION

Four primers were tested for their ability to detect DNA polymorphism in the fungal isolates under investigation. Each has developed different fingerprints for the tested isolates; however, isolates designated as Fov showed a very high degree of similarity with each primer. On the other hand, isolates designated as Fo (1-8) did not show similar level of similarity or homogeneity as they showed much variation in RAPD fingerprints.

Primer no. 1 (CGTGCGGGAA) detected a number of polymorphic markers in the tested isolates (Fig. 1A and 1B). Isolates representing Fov (9 - 15) developed one cluster with an overall similarity of 88.53 %. It was obvious that isolates 9, 12 and 15 showed a 100 % similarity and the three shared a 92.31 % similarity with isolates 11. Isolates 10 and 14 showed 100 % similarity and both shared 93.33 % similarity with

isolate 13. On the other hand, isolates designated as Fo (1 – 8) showed more diversity and were not all closely related as evidenced by the visualized banding patterns. They were separated in three clusters, where isolates 7, 3 and 2 expressed 47.22 % similarity, whereas isolates 4 and 8 have 54.55 % similarity. Isolates 1, 6 and 5 showed 61.57 % similarity with a cluster of Fov isolates. Progressively, diminishing similarity levels were observed between the previously mentioned subcluster and an overall similarity as low as 17.29 was detected for all 15 isolates tested.

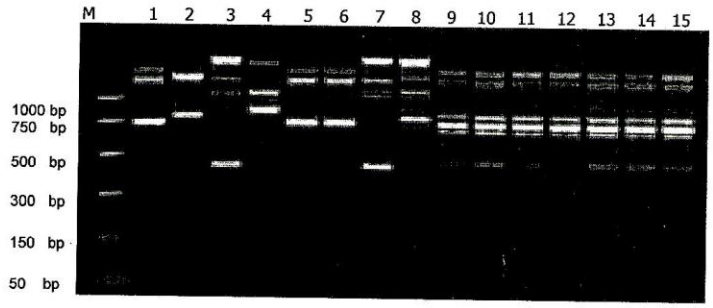

Fig. 1 A. RAPD banding patterns fingerprints for *Fusarium oxysporum* isolates and *F. oxysporum* f. sp. *vasinfectum* isolates obtained with the primer no. 1 and electrophoresed on 2 % agarose gel. Lanes from left to right were (M) DNA marker (1000-50 bp), isolates (1-8) of *Fusarium oxysporum* and lanes (9-15) for isolates of *F. oxysporum* f. sp. *vasinfectum*.

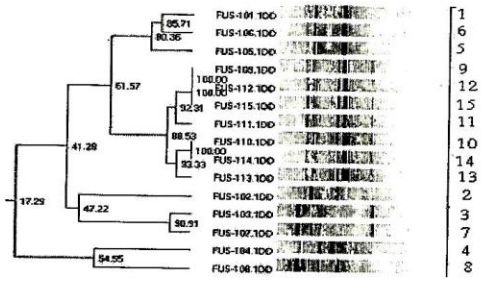

Fig. 1 B. Phenogram based on cluster analysis of RAPD banding patterns of *Fusarium oxysporum* isolates and *F. oxysporum* f. sp. *vasinfectum* isolates obtained by the primer no. 1 and electrophoresed on agarose gel. Shown in Fig.1 A.

Regarding the results obtained with primer no. 4 (AAGAGCCCGT), all the isolates of Fov were placed in one cluster with 99.10 similarity level. The isolates of Fo were grouped in three subclusters (Fig. 2A and 2B).

Isolates 1, 5 and 6 showed a 99.32% similarity level in one subcluster. The remaining isolates were placed in two subcluster where isolates 3 and 7 showed similarity level of 97.65% and isolates 2, 4 and 8 had 62.55 % similarity. The three subcluster are more closely placed compared with primer No. 1. Primer 4 showed a higher overall similarity level among all 15 isolates at 47.74 %.

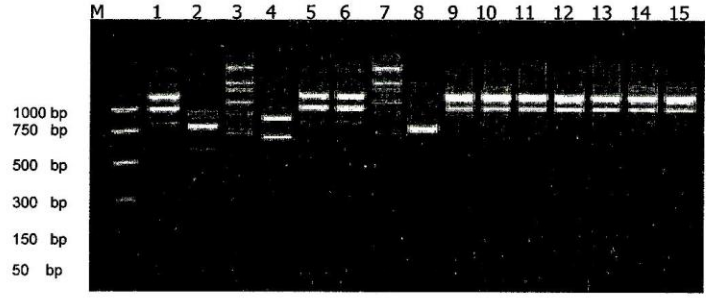

Fig. 2 A. RAPD banding patterns fingerprints for *Fusarium oxysporum* isolates and *F. oxysporum* f. sp. *vasinfectum* isolates obtained with the primer no. 4 and electrophoresed on 2 % agarose gel. Lanes from left to right were (M) DNA marker (1000-50 bp), isolates (1 - 8) of *Fusarium oxysporum* and lanes (9 - 15) for isolates of *F. oxysporum* f. sp. *vasinfectum*.

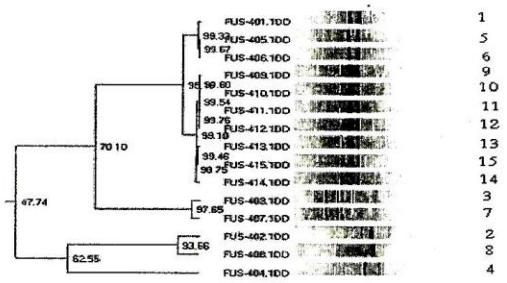

Fig. 2 B. Phenogram based on cluster analysis of RAPD banding patterns of *Fusarium oxysporum* isolates and *F. oxysporum* f. sp. *vasinfectum* isolates obtained by the primer no. 4 and electrophoresed on agarose gel shown in Fig. 2 A

With respect to primer no 5 (AACGCGCAAC), results show that all the isolates of Fov 9, 10, 11, 12, 13, 14 and 15 were placed in one cluster with similarity level 91.58 (Figs. 3 A. and 3 B.). The isolates of Fo were divided into three subcluster, the first one again included isolates nos. 1, 5, and 6 with SL 91.67%, the second subcluster encompassed the isolates nos. 2 and 4 at 85.90 % SL, whereas isolates 3, 7 and 8 clustered at 87.39 % similarity. The latter two subclusters showed 76.67 % similarity and clustered with Fov isolates with 67.57 % similarity. The overall similarity of the 15 isolates was 50.41 %.

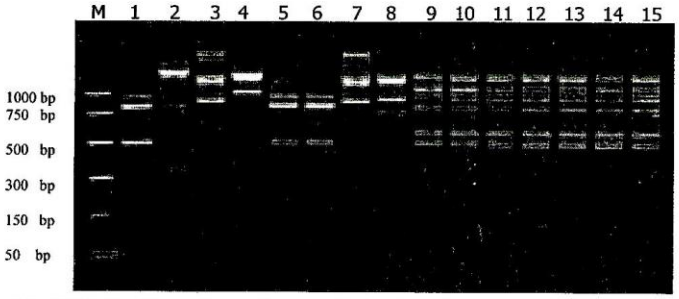

Fig. 3 A. RAPD banding patterns fingerprints for *Fusarium oxysporum* isolates and *F. oxysporum* f. sp. *vasinfectum* isolates obtained with the primer no. 5 and electrophoresed on 2 % agarose gel. Lanes from left to right were (M) DNA marker (1000-50 bp), isolates (1-8) of *Fusarium oxysporum* and lanes (9-15) for isolates of *F. oxysporum* f. sp. *vasinfectum*.

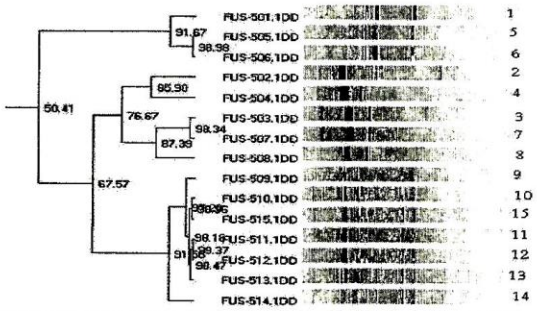

Fig. 3 B. Phenogram based on cluster analysis of RAPD banding patterns of *Fusarium oxysporum* isolates and *F. oxysporum* f. sp. *vasinfectum* isolates obtained by the primer no. 5 and electrophoresed on agarose gel shown in Fig. 3 A.

Primer no. 6 (CCCGTCAGCA) indicate that all the isolates of Fov were also placed in one cluster with similarity level 96.12 % Also, isolates 1, 5 and 6 of Fo were placed in separate subcluster with similarity level 98.04 % The latter clustered with isolates of Fov at a similarity level of 92.35 %. Isolates 3 and 7 of Fo showed 95.59 % similarity, whereas isolates 4 and 8 were 91.83 % similar. The two subclusters of Fo formed a cluster with 87.25 % similarity. Still isolate no. 2 showed a more distinct relationship with other Fo isolates; however, it showed a 69.19 % similarity to the cluster including Fov and isolates 1, 5 and 6 of Fo.

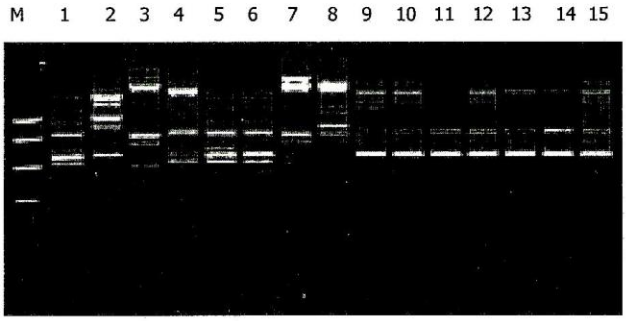

Fig. 4 A. RAPD banding patterns fingerprints for *Fusarium oxysporum* isolates and *F. oxysporum* f. sp. *vasinfectum* isolates obtained with the primer no. 6 and electrophoresed on 2 % agarose gel. Lanes from left to right were (M) DNA marker (1000-50 bp), isolates (1-8) of *Fusarium oxysporum* and lanes (9-15) for isolates of *F. oxysporum* f. sp. *vasinfectum*.

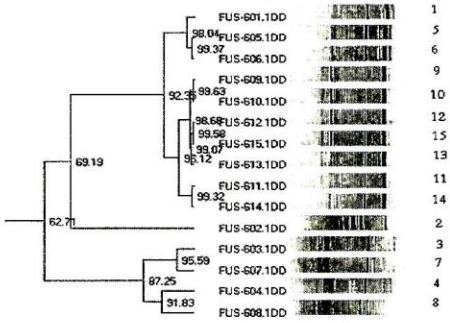

Fig. 4 B. Phenogram based on cluster analysis of RAPD banding patterns of *Fusarium oxysporum* isolates and *F. oxysporum* f. sp. *vasinfectum* isolates obtained by the primer no. 6 and electrophoresed on agarose gel shown in Fig . 4 A .

The data of the four primer show that the isolates of Fov always separated in one individual subcluster, indicating a high homogenicity within the genotype of isolates tested. These primers can be used in the differentiation between Fov and Fo. Regardless of the arrangement of Fov isolates within every subcluster, due to the high similarity level, they could be easily recognized by overall similarity levels ranging between 88.53 and 99.76% with the different primers, independent of their pathogenic potential (40 – 67.5 %). The results confirm that Fov populations in Egypt belong to one race ,i.e. race3 (Kerstin *et al.*, 2001). Accordingly, the design of a specific primer can be suggested for the rapid detection of Fov. This is in agreement with the results of Grajal-Martin *et al.* (1993), Assigbetse *et al.* (1994), Manulis *et al.* (1994), and Migheli *et al.* (1998), with other formae specialis.

The isolates nos. 1, 5 and 6 of Fo always separate in an individual subcluster with the four primers. They show a higher similarity; however, such a clustering could not be compared to the rest of the Fo isolates or correlated with the pathogenic ability of such isolates. They showed a variation range between 52.5 and 65 %. Larger number of isolates of different geographical origin and pathogenic potential may help uncovering certain relationships between fingerprinting and other characteristics in this group.

Present results suggest the suitability of RAPD for rapid molecular characterization of Fov isolates. On the other hand, it showed variation among some Fo isolates causing damping-off, which could not be explained on the basis of pathogenicity characteristics. Other factors may be involved in such a variation. The noticed clustering and separation of Fov in a more homogenic pattern, confirm that the populations of Fov in Egypt represent a single race of the pathogen which show a high degree of stability although different isolates may exhibit some degree of variation in pathogenic potential. Such a conclusion was recently reached by Abd-Elsalam *et al.* (2004) using RAPD and AFLP DNA tests. .

The results of the present work may suggest the feasibility of a preliminary RAPD testing of isolates prior to greenhouse phase, which is a laborious, time consuming, and subject to possible temperature variation.

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أستخدام التضاعف العشوائي للحامض النووى لتمييز فطر الذبول الفيوزاريمى فى القطن

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يعتبر ظهور أعراض الذبول العامل الرئيسى فى تعريف عزلات فيوزاريم أوكسيسبورم فازنفكتم. ويستهلك إختبار القدرة المرضية المستخدم فى التعريف كثيراً من الوقت كما أنه يتأثر بالتغير فى درجات الحرارة . ولذلك تم استخدام اختبار التضاعف العشوائى للحامض النووى فى التفرقة بين هذة العزلات وعزلات فيوزاريم أوكسيسبورم المسببة لسقوط البادرات وذلك عن طريق أستخدام اى من البادئات التالية :

1: (CGTGCGGGAA)-3 أو 4: (AAGAGCCCGT)-3

5: (AACGCGCAAC)-3 أو 6: (CCCGTCAGCA)-3

وقد وجد أن هذه الطريقة حساسة ومثالية ولها جانب تطبيقى وموفرة للوقت بجانب أختبار القدرة المرضية فى الصوبة الزراعية . وأظهرت العزلات المسببة للذبول درجة عالية جداً من التشابه وذلك عكس عزلات فيوزاريم أوكسيسبورم المختبرة والمسببة لتساقط البادرات والتى أظهرت أنخفاضاً فى التشابه الكلى لكل نتائج الأختبار العنقودى .