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Genetic diversity among *Fusarium oxysporum* f. sp. *vasinfectum* isolates revealed by UP-PCR and AFLP markers

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Summary. *Fusarium* wilt of cotton is responsible for significant yield losses worldwide. In this study we used UP-PCR and AFLP molecular marker techniques to evaluate and study the genetic similarity and phylogeny of 20 *Fusarium oxysporum* f. sp. *vasinfectum* isolates. On the basis of comparisons of genetic similarity matrices, AFLP markers were very similar to UP-PCR markers for estimating intra-specific genetic relationships. Dendrograms constructed with both AFLP and UP-PCR revealed that all accessions of *F. oxysporum* f. sp. *vasinfectum* isolates were grouped into the same cluster, in good agreement with the pathogenicity tests. The principal difference between the two techniques was that AFLP markers gave better resolution than UP-PCR for discriminating closely related Fusaria.

Key words: cotton, dendrogram, genetic similarity, PCR-based markers.

Introduction

Fusarium causing wilt of cotton was first isolated as a root rot pathogen (Woodroof, 1927; Colyer, 1988) that also caused delay in boll formation (Sparnicht and Roncardori, 1972). Cotton seed and seedling diseases have been described by Simpson *et al*. (1973), Watkins (1981), and Hillocks (1992). *Fusarium* wilt is caused by failure of the infected xylem to meet the water requirements of the plant (Hillocks, 1992).

Vascular wilt of cotton caused by *Fusarium oxysporum* f. sp. *vasinfectum* (Atk.) Snyd and Hans (Fov) occurs in all cotton-growing areas of the world with the exception of Turkey. The disease is particularly significant in south-eastern USA, Egypt, Sudan and China, where environmental conditions and sandy soils favor disease development (Hillocks, 1992). Until now, only one race, race 3 of Fov (=*F. vasinfectum* Atk. var. *egyptiacum* Fahmy) has been reported from Egypt (Fahmy, 1927). A determination of both host specificity and the genetic diversity of Fov populations is of great importance in breeding cotton for resistance. An assessment of the genetic diversity of *Fusarium* wilt of cotton is needed to determine whether races constitute genetically distinct groups, and to obtain molecular markers for differentiating them.

The UP-PCR fingerprinting technique is a useful tool for the characterization and grouping of fungal strains in order to explain their genetic relatedness (Lübeck *et al*., 1999). UP-PCR has been applied successfully for the identification of differences among populations of fungal pathogens, in-

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cluding *Fusarium* spp. (Bulat *et al*., 1996; Yli-Mattila *et al*., 1997a, b). Another method for finding discriminating DNA marker fragments is by amplified fragment length polymorphism (AFLP) analysis (Vos *et al*., 1995). For a wide range of taxa, including plants, fungi and bacteria, AFLP markers have ascertained cryptic genetic differences between strains or closely related species that were impossible to detect with morphological or other molecular systematic characters. AFLP therefore has a broad taxonomic applicability in a variety of taxa including bacteria (Huys *et al.,* 1996) and fungi (Majer *et al*., 1998). In previous studies, RAPD analysis was used to discriminate between Fov populations isolated from cotton (Assigabetse *et al*., 1994; Feng *et al*., 2000; Smith *et al.,* 2001). The aim of this study was to evaluate the usefulness of RAPD and other techniques to characterize genetic diversity within populations of Fov isolated from cotton-growing areas in Egypt.

Materials and methods

Pathogenicity test of *F. oxysporum* f. sp. *vasinfectum* on cultivar Giza 74

Twenty isolates of Fov were tested in the greenhouse on cultivar Giza 74, which has no known resistance to *Fusarium* wilt. The inoculum level of each isolate was 50 g fresh mycelium kg^{-1} soil, and each pot was planted with 10 seeds. The greenhouse temperature ranged from 22 to 32°C during the test. The percentage of wilted seedlings was recorded 45 days after planting. External morphological disease symptoms produced by the Fov isolates appeared 6 weeks following inoculation. They were typically vein clearing starting on the smaller vein. The internal anatomical symptom in the vascular system of infected plants was brown to black discoloration which was easily visible below the bark when the stem was cut. The pathogenicity index was scored as described previously by Aly *et al*. (2000).

Culture conditions

DNA was extracted from the twenty Fov isolates from different cotton-growing areas in Egypt. The isolates were maintained using the cryobiologic freezing method of Schnieder (1998). The isolates and a skim-milk solution were frozen according to a defined freezing programme using liquid nitrogen at the steam phase and stored at –70°C. The isolates were examined one and five months after cryobiologic freezing to determine the germinability of the spores and their pathogenic effect on host plants (incubation period, proportion of necrotic leaf and latent period).

Fungal DNA isolation

Five hundred mg fresh fungal mycelium (4 to 7 days old) of each isolate was scraped off Petri dishes and added to 1.5-ml microcentrifuge tubes. Total DNA was extracted basically as described by Bulat *et al*. (1998).

UP-PCR conditions

Amplification reactions were performed in 0.2-ml microcentrifuge tubes in a 25-µl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.8 mM NaCl, 3.5 mM $MgCl_2$, 0.1% Triton X-100, 0.4 mM dNTPs, 40 ng primer L45 (5'-GTAAAACGACGGCCAGT-3'), 1.0 U of Taq DNA polymerase (Promega, Mannheim, Germany), and 10 to 15 ng of genomic DNA. PCR amplification was performed in a Primus 96 HPL Thermal Cycler System (MWG-Biotech, Ebersberg, Germany) programmed for 30 cycles of denaturation at 94°C for 30 s (first denaturation step at 94°C for 3 min), annealing at 56°C for 70 s and polymerization at 72°C for 60 s, with a final extension step of 72°C for 5 min. The reaction tubes were held at 4°C following the final amplification cycle. Two µl UP-PCR products (1/10 of the total reaction) were electrophoresed in 2% agarose gel at 150 V with TAE buffer for 50 min according to Lübeck *et al*. (1999). Gel was stained with ethidium bromide, photographed and scanned using American Advanced Biotechnology software (AAB Program) (Fullerton, CA, USA).

AFLP assays

AFLP fingerprinting was performed as described by Vos *et al*. (1995) with some minor modifications (Abd-Elsalam *et al*., 2002). The restriction-ligation mixture contained 500 ng of genomic DNA, 1 µl of 10× T4 DNA ligase buffer, 1 µl of 0.5 M nuclease-free NaCl, 0.5 µl of a 10-mg ml^{-1} bovine serum albumin (New England Biolabs, Hitchin, Herts, UK) solution, 1 µl each of the double-stranded *Eco*RI and *Mse*I adapters (Perkin-Elmer,

Gaithersburg, MD, USA), and 1.6 µl of an enzyme mixture consisting of 1 U of T4 DNA ligase (Roche, Mannheim, Germany), 5 U of *Eco*RI (Roche), and 1 U of *Tru*I (Roche), an isoschizomer of *Mse*I. The restriction-ligation mixture was incubated at 37°C for 3 h and then diluted with 0.1 M TE buffer (20 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) to a final volume of 200 µl. The following combinations of AFLP primer pairs with three selective nucleotides each (bold letters) were used for the amplification of the *Eco*RI-*Mse*I fragments: (i) *Eco*RI-TCA (5'-GAC TGC GTA CCA AT**T CA**) and *Mse*I-AAA (5'-GAT GAG TCC TGA GT**A AA**), (ii) *Eco*RI-TCC (5'-GAC TGC GTA CCA AT**T CC**) and *Mse*I-AAC (5'-GAT GAG TCC TGA GT**A AC**).

DNA amplification was performed on a 20-µl mixture consisting of 4 µl of the diluted restriction-ligation mixture, 1 µl of *Eco*RI and *Mse*I preselective amplification primer pairs, and 15 µl of AFLP core mixture (Perkin-Elmer). PCR amplifications were performed with a Primus 96 HPL Thermal Cycler System using the following protocol: an initial step consisting of 72°C for 2 min, 20 cycles consisting of 94°C for 1 s, 56°C for 30 s, and 72°C for 2 min, and a final extension step consisting of 72°C for 7 min. The PCR products were stored at –20°C until electrophoresis. The amplified fragments were separated electrophoretically in a denaturing 4% polyacrylamide sequencing gel with 8 M ultrapure urea. After electrophoresis, AFLP fingerprints were detected by a modification of the silver staining method of Creste *et al*. (2001).

Computer analysis

The experimental design of all greenhouse studies was completely randomized with five replications. Duncan´s multiple range test was used to compare treatment means. Some percentage data were transformed into arc sin angles or square roots before carrying out ANOVA to produce approximately constant variance.

Cluster analysis of AFLP patterns was done by analyzing the scanned profiles with the AAB Program. Similarity between fingerprints was calculated with the Pearson product moment correlation coefficient (r). Cluster analysis was performed by the unweighted pair group method with average linkages (UPGMA). The correlations between AFLP and UP-PCR distance matrices were analyzed using Mantel's (1967) test.

Results

Pathogenicity test

All 20 Fov isolates tested under greenhouse conditions were pathogenic to cotton seedlings of Giza 74. Fov isolates 12, 15, 18 and 20 were highly pathogenic causing 96–100% mortality. Control plants did not develop any symptoms. The results of the pathogenicity tests are given in Table 1.

UP-PCR analysis

UP-PCR based genetic similarity (GS) analysis clustered nineteen Fov isolates into the first main cluster (Fig. 1), the genetic similarity ranging from 68 to 92.8%. Fov isolates 18 and 20 showed high genetic relatedness (92.8%). Only Fov isolate 19 was placed in the second main cluster, with a genetic similarity of 61.8%. There was no association between clustering in the UP-PCR dendrogram and the geographic origin of the isolates. These results suggest that with a few exceptions UP-PCR gave clear-cut patterns distinguishing between the pathogenicity of Fov isolates.

AFLP analysis

In the first experiments, primer combinations with different numbers of selective bases at the 3' end were compared to determine which gave the most information. *Eco*RI+3 primers were combined with *Mse*I+3 primers; a large number of defined bands was scored. All further experiments were therefore performed with *Eco*RI+3 primers in conjunction with *Mse*I+3 primers. Out of 60 primer combinations, four were selected for their reliable banding pattern (Table 2). An example of two combinations is shown in Fig. 2.

Between 35 and 89 bands were amplified with each of the four AFLP primer combinations. Among Fov isolates, similarities ranged from 76 to 100%. A dendrogram of the AFLP primer combinations separated the Fov isolates into two main clusters. The first cluster included ninteen Fov isolates. Fov Isolates 1, 2, 12, 15, 18 and 20 were genetically identical.

The second cluster contained only one isolate (Fov19). There was no correlation between clustering in the AFLP dendrogram and the geographic origin of the isolates, but a correlation between pathogenicity and the grouping of Fov isolates was found. For example, Fov isolates 18 and 20, both with a GS of 100% came from the same location.

Table 1. Geographic origin of the 20 *Fusarium oxysporum* f. sp *vasinfectum* isolates and pathogenicity tests determined in this study.

Isolate No.	Location	Wilt symptoms [a]			
		Healthy (%)	Dead (%)	Morphological (%)	Anatomical (%)
1	Beheira	26.0 df * [b]	11.0 ef	64.5a*	0.0 c
2	Minufiya	34.2 b-e *	65.8 b*	0.0 e	0.0 c
3	Minufiya	26.3 c-e *	48.9 b-d*	24.8 a-d*	0.0 c
4	Fayium	17.5 e-g *	55.8 bc*	18.3 c-e*	8.3 b*
5	Fayium	47.4 b-d*	22.4 de*	29.1 bc*	0.0 c
6	Beheira	26.6 c-e*	58.3 bc*	15.0 c-e	0.0 c
7	Minufiya	40.0 b-e*	45.0 b-d*	15.0 c-e	0.0 c
8	Fayium	8.6 f-h*	53.2 b-d*	25.4 b-d*	12.7 a*
9	Daqahliya	54.2 bc*	35.8 b-d	10.0 de	0.0 c
10	Beheira	62.0 b*	25.2 c-e*	12.7 c-e	0.0 c
11	Sharqiya	33.7 b-e*	35.7 b-d*	26.5 b-d*	4.9 bc
12	Gharbiya	3.1 gh*	96.9 a*	0.0 e	0.0 c
13	Minufiya	30.3 b-e*	67.0 b*	0.0 e	0.0 c
14	Assuit	4.2gh*	49.6 b-d*	46.3 ab*	0.0 c
15	Assuit	0.0 h*	100 a*	0.0 e	0.0 c
16	Minufiya	26.6 c-e*	48.9 b-d*	15.0 c-e	9.6 c
17	Dumyat	17.5 e-g *	54.9 bc*	19.3 c-e*	8.3 b*
18	Daqahliya	0.0 h*	100 a*	0.0 e	0.0 c
19	Fayium	8.6 f-h*	55.8 bc*	25.4 b-d*	11.5 a*
20	Daqahliya	0.0 h*	100 a*	0.0 e	0.0 c
21	Control	100.0 a	0.0 f	0.0 e	0.0 c

[a] Percentage data were transformed into arc sine angles before performing analysis of variance.
[b] Values followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.
* The value shows a significant difference from the control.

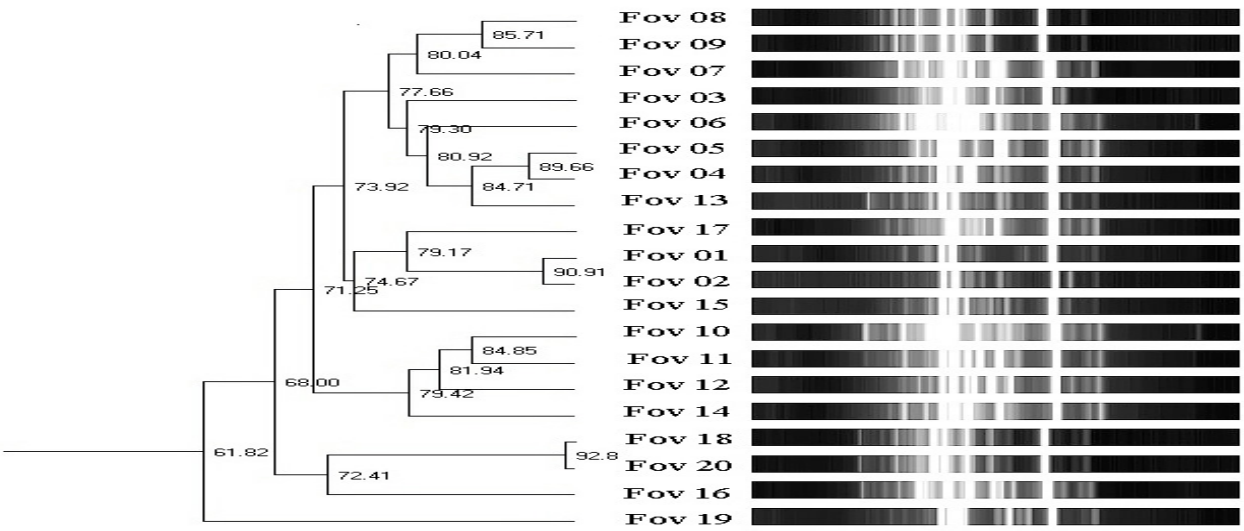

Fig. 1. Digitized UP-PCR patterns and dendrogram for 20 isolates of *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) using agarose gel (2.0%) stained with ethidium bromide. The dendrograms were constructed with the UPGMA clustering method.

Comparison of AFLP and UP-PCR genetic similarities

In order to compare the two clusters, each generated by one of the marker systems, a Mantel matrix-correspondence test was carried out on the basis of comparisons of co-phenetic values (Fig. 3). The overall representation of relationships by cluster analysis was similar for the two marker systems with high correlations among Fov isolates (r=0.64).

Table 2. Number of polymorphic markers generated per primer pair combination in *Fusarium oxysporum* f. sp. *vasinfectum* populations

Primers	E-TCA[b]	E-TCC
M-AAA[a]	12–15	14–17
M-AAC	10–14	12–14

[a] M indicates *Mse*I primer 5'GAT GAG TCC TGA GT +NNN 3'
[b] E indicates *Eco*RI primer 5' GAC TGC GTA CCA AT +NNN 3'

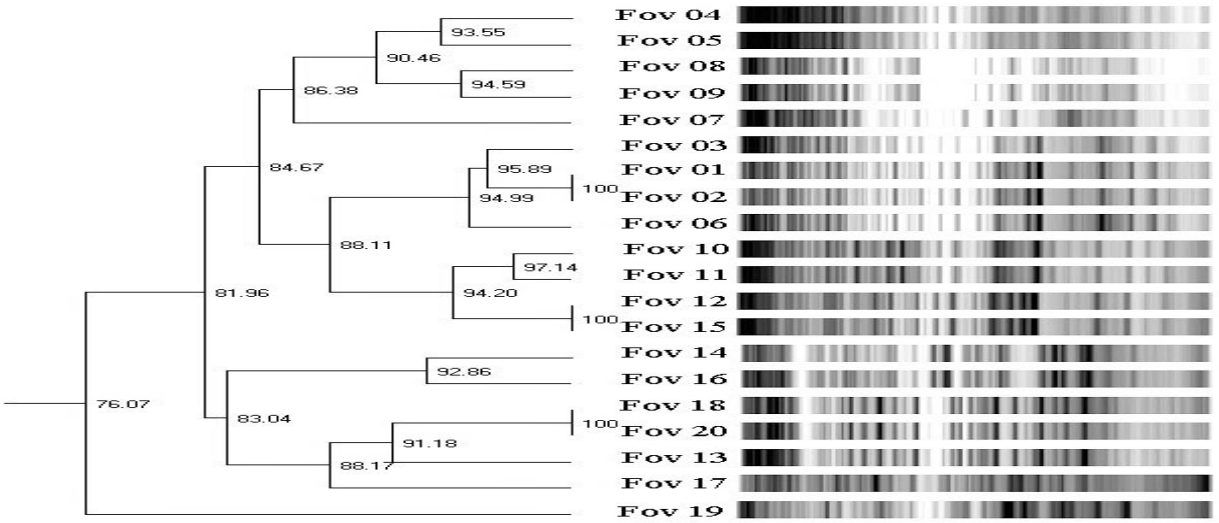

Fig. 2. Digitized AFLP patterns and dendrogram for 20 isolates of *Fusarium oxysporum* f. sp. *vasinfectum* using primer combination E-TCC and M-AAA on polyacrylamide gel (4.0%) stained with silver stain. The dendrograms were constructed with the UPGMA clustering method.

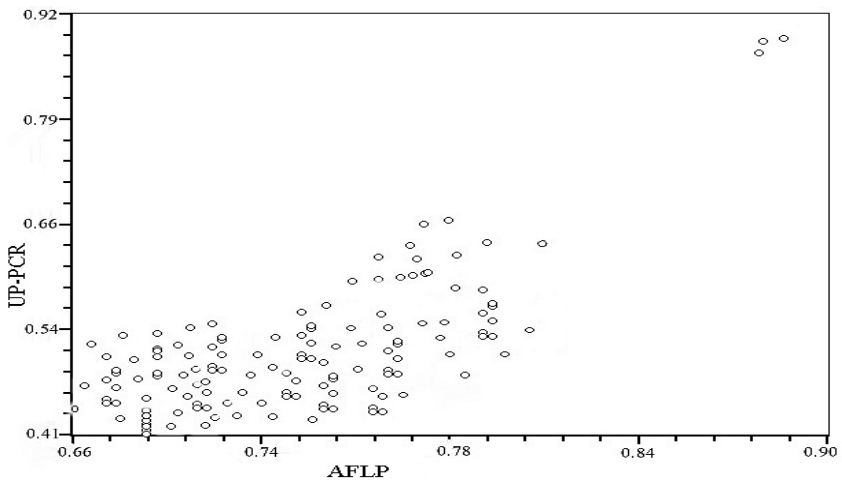

Fig. 3. Comparison of genetic similarity estimates from UP-PCR and AFLP using the Mantel test.

Discussion

Molecular marker analysis can be useful for determining the relationship between genotypes and population data such as morphology, disease susceptibility and geographical location. In our experiment, we conducted a pathogenicity test on the cotton cultivar Giza 74 under conditions very favorable for unrestricted isolate development. The soil was sterilized, temperature was optimal most of the time, inoculum density was relatively high and the test cultivar was highly susceptible. All isolates were pathogenic to seedlings of Giza 74.

Twenty Fov isolates were characterized by the UP-PCR and AFLP techniques combined with pathogenicity assays. UP-PCR was used to further differentiate isolates of the groups revealed by molecular typing. UP-PCR is an effective tool for the rapid intraspecific typing of strains at the molecular genetic level and for the study of *F. oxysporum* populations (Bulat *et al*., 1995).

According to Lübeck *et al*. (1999), UP primer L45 generates fingerprints with a relatively great number of bands. Four primer combinations were used, which produced 267 bands, 215 (80.5%) of which were polymorphic. Nineteen isolates had a unique genotype, without clear correlation with their geographical origin. Similarly, Leissner *et al*. (1997) used AFLP fingerprinting to study 18 different *F. graminearum* strains, fifteen of which showed a high degree of similarity in the banding patterns. O'Neill *et al*. (1998) found less than 70% similarity between *Fusarium udum* and *F. oxysporum* formae speciales pathogenic to coca, cowpea, and tomato.

The findings showed a clear relationship between UP-PCR and AFLP profiles, and the pathogenicity tests for Fov isolates 12, 15, 18 and 20. A high GS between isolates 18 and 20 might explain why some UP-PCR and AFLP markers were shared. A similar case was reported by Assigbetse *et al*. (1994), who suggested that RAPD markers were a quick and reliable alternative for assigning Fov isolates to their respective pathogenicity group. Woo *et al*. (1996) also found that some pathogenically diverse isolates of *F. oxysporum* f. sp. *phaseoli* had very similar banding patterns. Smith *et al*. (2001) characterized two types of *F. oxysporum* f. sp. *vasinfectum* by RAPD analysis and pathogenicity tests.

The AFLP technique using silver staining to visualize the bands has been successfully applied to the molecular characterization of *Fusarium* spp., and there was a clear relationship between AFLP groups and morphological characters (Abd-Elsalam *et al*., 2002). For UP-PCR and AFLP markers, a high reproducibility in dendrogram topologies was obtained, with only a few differences in a small number of accessions, such as Fov 19. Also, both marker types enabled the differentiation between Fov isolates. No clear-cut relation between geographical origin and particular Fov groups became evident. In a study of *F. avenaceum* Yli-Mattila *et al*. (1996) also found no correlation between isozymes, RAPD-PCR and UP-PCR patterns on the one hand, and geographic origin or particular host plants on the other. In a RAPD-PCR analysis of *F. oxysporum*, Assigbetse *et al*. (1994) reported a correlation between GS and geographic origin.

In conclusion, the AFLP and UP-PCR methods provide similar results, but AFLP was faster and more robust than UP-PCR. The AFLP markers also showed a higher degree of resolution for discriminating closely related fungi and this technique appears to be suitable for studying genetic diversity among *Fusarium* isolates.

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