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Diversity of *Fusarium oxysporum* infecting cotton seedlings in Egypt

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ABSTRACT

A combined study of pathogenicity, mycelial compatibility, chemotaxonomic and molecular analyses was conducted to reveal the genetic variability in isolates of *Fusarium oxysporum* collected from cotton seedlings in Egypt. The results derived from these techniques were corroborative and suggested a considerable genetic diversity among isolates. However, the extent of diversity differed from analysis to another. The virulence of the isolates showed a wide range of variation ranged from 26.6 to 80%. The cluster analysis of mycelial compatibility data indicated no relationship between mycelial compatibility groups and virulence. The high percent of polymorphism at both chemotaxonomic and molecular levels suggested the complex evolution pattern and confirmed the polyphyletic origin of *F. oxysporum*. In addition, the partial association between the loci generated by one of the used primers and mycelial compatibility groups suggested that some mycelial compatibility groups were clonally derived.

KEYWORDS

Pathogenicity;
MCGs;
Soluble proteins;
RAPD;
Genetic diversity.

INTRODUCTION

Cotton (*Gossypium barbadense* L.) is one of the most fiber and field crops in Egypt[1-3]. Egyptian cotton has an extra long-staple in the world. It has long been considered as the most important economic crop in Egypt.

Seedling diseases are major problem in cotton production areas, being caused by one or multiple microorganisms especially fungi[4-6]. These diseases caused plant dead that make the growers replant the dead area, causing late maturity. Damping off is one of these devastated diseases that disseminates in Egypt where cotton is grown[7]. It causes seed rotting, seedling damping-off and decreasing cotton production. The most common soilborne fungi associated with seedling damping off is *Fusarium* spp. The estimated loss caused by *Fusarium* spp. is an average of 8.6 percent annually based on a range of five to 18 percent since 1989[7,8]. Loss estimates do not include the cost of replanting or losses due to lateness of replanted cotton.

In Egypt, the high frequency species isolated from infected cotton roots from different districts was *F. oxysporum*[9]. It represents about 52.7% of the pathogens separated from cotton seedlings suffered from post emergence damping-off, root rot and wilt diseases[10].

Vegetative compatibility groups (VCG;) are a natural way of subdividing fungal populations, making genetic variation between the individuals of the populations. There are two mechanisms favor exchange of

nuclear material and organelles between incompatible strains. These mechanisms are known as conidial anastomosis and hyphal anastomoses. In conidial anastomosis, conidial anastomosis tubes are formed between conidia within acervuli. This mechanism was observed during *C. lindemuthianum* conidiogenesis[11]. On the other hand, hyphal anastomoses was observed among vegetative compatible isolates and "lead to the formation of vegetative heterokaryons (i.e., cells containing two genetically different nuclei), which have been characterized as the first stage of the parasexual cycle"[12-15]. "Haploid nuclei in the heterokaryotic mycelium fuse together and form diploid nuclei. Through successive processes of chromosome non-disjunction, the latter give rise to paternal haploids or recombinants"[12,16]. The viability of heterokaryons is regulated by multiple vegetative incompatibility loci, named *vic* (for vegetative incompatibility) or *het* (for heterokaryon incompatibility), so that only compatible strains, believed to be clonally related, may produce stable heterokaryons among themselves[13]. 'The *het* loci be-have as if they were part of a recogni-tion system that enables individuals to identify each other and to differentiate themselves from each other. 'The *het* loci can delimit the pathotypes of asex-ual phytopathogenic fungi, as occurs in the genus *Fusarium*[17].

Biochemical and molecular markers are being increasingly used to characterize fungal plant pathogen populations, including *Fusarium oxysporum* populations[18-22]. They are often versatile and highly informative tools for fungal pathogen identification at sub-specific taxa *i.e. formae speciales* or physiologic races and diagnosis[23-25] and for populations genetic studies[26-28]. They can be used to evaluate levels of genetic diversity, systematic relationships and as an adjunct to morphological criteria in taxonomy[29-33].

Our objectives in this study were to analyze the diversity and genetic relationships of *F. oxysporum* isolates collected from cotton fields in a number of districts in Egypt using pathogenicity, vegetative compatibility, electrophoretic patterns of the soluble proteins and random amplification polymorphism DNA (RAPD).

MATERIALS AND METHODS

Fungi isolation

Fusarium oxysporum isolates were isolated from infected tissues of 20 stems and roots of cotton seedlings collected from fields in each of district (TABLE 1). The stems and roots were washed in tap water for 1 minute, and cut into small pieces, 3-5 mm thickness. The pieces were surface sterilized by soaking in 70% ethanol for 30 seconds, and placed on filter paper for 2 min to dry out. Four sterilized pieces were transferred to 9 cm diameter Petri dishes containing 20 ml of potato dextrose agar medium (1/4 strength PDA)[34]. The plates were incubated at 25ºC for 7 days. Once colonies of *Fusarium oxysporum* were established, they were transferred to slants of 1/4 strength PDA made for maintenance. All isolates were maintained over peri-

TABLE 1 : Virulence of *Fusarium oxysporum* isolates on cotton (cv Giza 89) damping-off.

Isolates	District (Locality)	Damping-off*		Seedling survival (%)
		%Pre	%Post	
F1	El-Beheira (Tahreer)	40.0	0.00	60.0
F2	El-Gharbia (Kotor)	53.3	6.60	40.1
F3	Kafr el Shiekh (Kafr el Shiekh)	40.0	13.3	46.7
F4	El-Dakahlian (Bilqas)	46.6	6.60	46.8
F5	Damietta (Kafr Saad)	80.0	6.60	13.4
F6	El-Sharkia (El-Salehia)	46.6	6.60	46.8
F7	El-Menufia (Ashmon)	33.3	13.3	53.4
F8	El-Kalyubia (Tokh)	26.6	6.60	66.8
F9	Giza (El-Aayat)	20.0	20.0	60.0
F10	Beni Suef (Beba)	66.6	0.00	33.4
F11	El-Fayum (Abshaway)	73.3	0.00	26.7
F12	El-Minya (Mattai)	53.3	13.3	34.0
F13	Asyut (Badarei)	33.3	0.00	66.7
F14	Sohag (Maragha)	53.3	6.60	40.1
F15	North Sinai (El-Aresh)	26.6	6.60	66.8
F16	El-Sharkia (El-Ebrahimia)	40.0	0.0	60.0
Control		0.00	0.00	0.00
L.S.D. at 0.05		6.92	4.84	

***Mean of five replications**

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ods of 3 months on slants at 5ºC in a refrigerator for more frequent use.

Identification

The isolated *Fusarium oxysporum* from cotton was identified according to the descriptions of *Fusarium* by Booth[35], Nelson *et al*.[36] and confirmed by the identification unit in Mycological Research and Disease Survey Department, Agricultural Research Center, Giza, Egypt.

Pathogenicity test

Sixteen isolates of *F. oxysporum* were tested under the greenhouse conditions for their pathogenic potentialities on susceptible cotton cultivar (Giza 89), obtained from the Agriculture Research Center, Giza, Egypt

Substrate for growth of the tested isolates of *Fusarium* was prepared in 500 ml glass bottle contained 100 g of sorghum grains, 50 g sand and 80 ml of tap water[9]. Contents of each bottle were autoclaved for 30 min. Isolate inoculum, taken from one week old culture grown on potato dextrose agar (PDA), was aseptically introduced into the bottle and allowed to colonize sorghum for 3 weeks. Clay pots were sterilized by immersing them in 5% formalin solution for 15 min, then left for 2 days to insure a complete evaporation of formalin. Pathogenicity test was carried out by using autoclaved clay loam soil for cotton. Seeds of cotton were surface sterilized by submerged in solutions of 20-30% commercial Clorox[(5.25%) sodium hypochlorite NaOCl] for 3 min, then washed several times in sterilized distilled water and dried between folds of sterilized filter paper.

Batches of soil were infested separately with inoculum of each isolate at the rate of 10 g/kg of soil[10,37]. Infected soil was dispersed in 15 cm diameter clay pots, with a number of five pots for each isolate. Each pot was planted with 5 seeds of cotton cultivar's Giza 89.

In the control treatment, non-infested sterilized sorghum grains were mixed thoroughly with soil at the rate of 10g/kg of soil. The prevailing temperature during pathogenicity tests was 30 ± 2ºC and seedlings were watered when necessary.

The percentage of disease incidence in cotton was measured as percentage of each of pre- and post-emergence damping-off after 7 and 14 days of sowing, respectively.

Vegetative compatibility

Vegetative compatibility was determined as described earlier[38]. Two inoculums plugs (5mm diameter) were placed in Petri dishes of 9 cm diameter containing PDA medium and incubated at 28°C in the dark for 14 days. Pairs of isolates were assessed after hyphal growth formation for compatible or incompatible reactions. Compatible reactions were recorded if the two colonies merged without forming a dark line or a strip of thin mycelium, and hyphal formation was continuous. Incompatible reactions were recorded when a reaction line formed between the colonies[39]. Incompatible reactions were almost invariably observed as a dark line or as a halted reaction where hyphal growth stopped as they approached each other resulting in a "stand off" but were not compatible with others.

Whole cell protein extraction

The tested isolates were incubated at 25ºC for 6 days on liquid PD medium (1/4 strength). The growing mycelia were harvested by filtration through cheesecloth, washed with distilled water several times, dried on filter paper, and stored at –80ºC for further use. Two grams of the frozen mycelia were ground with a mortar in liquid nitrogen to a fine powder[40-42]. The ground sample (50 mg) was extracted with 0.2 ml of extraction buffer [0.6 mL 1M Tris- HCl (pH 6.8), 5 mL 50% glycerol, 2 mL 10% SDS, 0.5 ml 2-mercaptoethanol, 0.9 mL H_2O].

Electrophoretic analysis of whole cell proteins by SDS-PAGE

The extracted proteins in the previous step were electrophoretically analyzed on 12% SDS-PAGE[43-46] and stained with silver nitrate[47]. To ensure that data of the whole cell proteins analysis was consistent and convinced, the whole cell proteins analysis was repeated three times.

Genomic DNA extraction

Three to four mycelial plugs (each 4 mm in diameter) from PDA cultures were transferred to flasks containing 150 ml of potato dextrose broth, which were incubated at 25±1°C on orbital shaker (125 rpm) for 4 days. 50 mg of mycelial growth was used to extract genomic DNA using Qiagen Fast Cycling PCR Kit for DNA extraction. The frozen mycelia were ground to a

fine powder in liquid nitrogen. The extracted DNA was dissolved in 100 µl of elution buffer. The concentration and purity of the obtained DNA was determined by "Gen quanta" system-pharmacia Biotech. The purity of the DNA for all samples was between 90-97% and the ratio between 1.7-1.8. Concentration was adjusted at 6 ng/µl for each sample before amplification with the RAPD.

RAPD analysis

Genomic DNA from *Fusarium oxysporium* was amplified by the RAPD using Fifteen arbitrarily chosen decamer primers (OPA-01, 02, 03, 04, 05, 06, 07, 08, 09, 10, 11, 12, 13, 14, 15) from Operon primer kit (Operon Technology Inc, USA) to determine if banding patterns produced by the arbitrary amplification could differentiate between the isolates. The primers OPA-01, OPA-03, OPA-05, OPA-08, and OPA-11 were selected among 15 primers tested on the basis of reproducible bands obtained. Preliminary amplification was conducted to determine the optimal concentration of the component in the PCR reaction mixture. The most intense bands were considered for the analysis. PCR amplifications were performed in a total volume of 20 µl containing 20 ng genomic DNA, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris/ HCl (pH 8.3), 200 µM dATP, dCTP, dGTP and dTTP, primer 100 ng, and 1U of Taq DNA polymerase (Promega Corp. Madison, WI). Each reaction was overlaid with 1 drop of mineral oil. PCR was carried out in PTC 100 programmable thermo cycler (MJ Research, Water Town, MA, USA). The program included an initial denaturation at 94°C for 4min, 40 cycles with denaturation at 94°C for 1 min, annealing 56°C for 1 min, an extension at 72°C for 2 min, and a final extension at 72°C for 7 min. Negative controls (no template DNA) were used for each set of experiment to test for the presence of nonspecific reaction. All experiments were repeated at least three times. The PCR products were electrophoresed on 1.2% agarose gel using 0.5X TBE buffer, stained with ethidium bromide and visualized under UV and photographed.

Data analysis

The data generated from mycelial compatibility, whole cell proteins and RAPD analyses for each isolate were compared on the basis of the presence (1) versus absence (0) of vegetative compatibility reaction, protein band and RAPD products of the same electrophoretic mobility. Relative relatedness among isolates was determined. Pairwise comparison was made between all isolates and the values used to generate a similarity matrix[48]. A dendrogram representing phenetic relationship between the isolates was constructed from the matrix of dissimilarities by the unweighted pair-group method algorithm (UPGMA). All calculations were conducted using the computer program NTSYS-pc. analysis.

RESULTS

Cotton seedlings artificially infected with the studied isolates showed damping-off symptoms appeared on cotton seedlings naturally infected with *F. oxysporium.* Root and stem tissues become discolored, turn brown internally and then rot. After emergence, necrotic lesions usually appear on the hypocotyls near the soil surface. However, the pathogenic capabilities of these isolates were different (TABLE 1). The pre-damping-off and post-damping-off percentages ranged from 20.0 to 80.0% and 0.0 to 20.0% respectively. The percentage of survival seedlings was between 13.4 and 66.8. The highest disease incidence was reported for the isolates F5 (80.0%) and F11 (73.3%) and the lowest was for F15, F8 and F9, see TABLE 1. The variation in disease incidence between different isolates was significant, as indicated by LSD values.

The 16 isolates of *Fusarium oxysporum* were subjected to study of vegetative compatibility. The results of vegetative compatibility illustrated in TABLE 2. The data indicated that all isolates were self-compatible. The application of UPGMA clustering produced 5 mycelial compatibility groups (MCGs) (Figure 1), with genetic distance ranged from 0.08 to 0.45. Group 4 includes with of the isolates. Some of these isolates had high pathogenicity and some other had low pathogenicity. The lowest pathogenic isolates (F9 and F15) were connected with the highest pathogenic ones (F5 and F11), at genetic distance of 0.23. The two highest pathogenic isolates were closely related, had a genetic distance of about 0.1.

The whole cell proteins of the studied isolates of *Fusarium oxysporum* were electrophoreticaly analyzed on SDS/PAGE (Figure 2). The electrophoretic patterns of the whole cell proteins of the studied isolates showed

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TABLE 2 : Mycelial compatibility of *F. oxysporum* isolates

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16
F1	+	-	-	-	-	+	+	+	-	-	-	-	-	+	+	+
F2	-	+	-	-	-	-	-	+	-	+	-	-	-	+	+	-
F3	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-
F4	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	+
F5	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
F6	-	-	-	+	-	+	-	-	-	-	+	-	+	-	-	+
F7	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
F8	+	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-
F9	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-
F10	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
F11	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-
F12	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-
F13	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+
F14	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
F15	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
F16	+	-	-	+	-	+	-	-	-	-	-	+	-	-	+	+

+ = compatible; - = incompatible

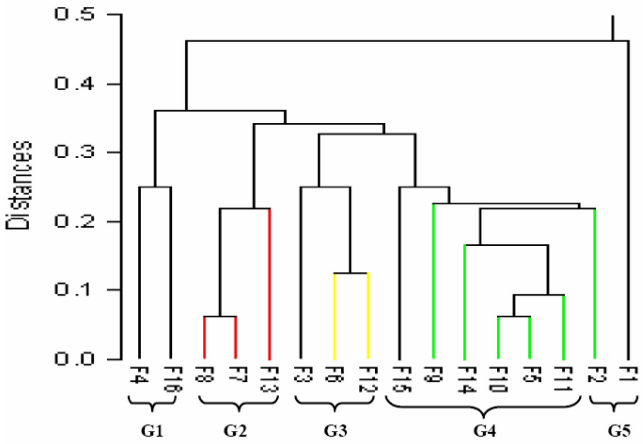

Figure 1 : Cluster analysis based on vegetative compatibility of *Fusarium oxysporum* isolates illustrating their mycelial compatibility groups.

a great number of polymorphic bands with molecular weights ranged from 14 to 99 KDa. Generally, there was no definite relation between the number or the intensity of soluble proteins bands and pathogenicity. Only, the most pathogenic isolates were characterized with protein band with molecular weight of 60 KDa (Figure 2). This band was also present in F9, one of the least pathogenic isolate. The cluster analysis based on the whole cell proteins data separated the isolates of *Fusarium oxysporum* into six clusters (Figure 3). Cluster 5 contains 37.5% of the isolates, including the most pathogenic ones.

The genomic DNA of 16 isolates of *Fusarium*

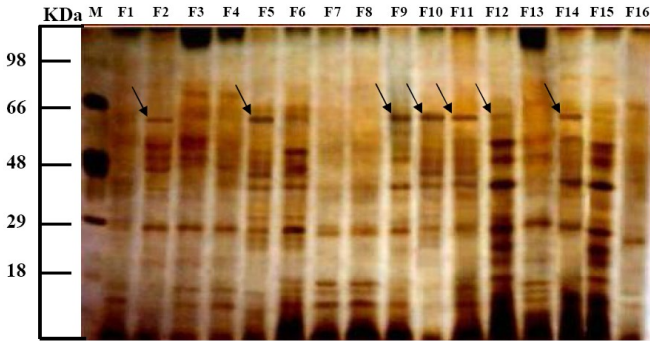

Figure 2 : SDS-PAGE analysis of *Fusarium oxysporum*. Arrows refer to the band with molecular weight of 60 KDs.

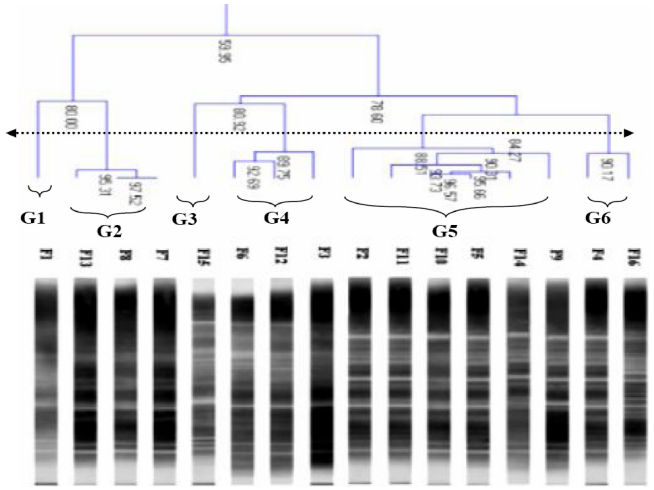

Figure 3 : Digitized patterns and dendrogram derived from SDS-PAGE analysis of *Fusarium oxysporum* isolates stained with silver nitrate. (mark the bands differentiating the clusters).

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oxysporum was amplified by RAPD analysis technique using 15 primers. Five primers (OPA-01, OPA-03, OPA-05, OPA-08, and OPA-11) out of the tested primers gave clear and reproducible amplification products. The profiles of the amplified bands by the 5 primers gave an extensive variation between the tested isolates, that is each isolate gave a unique profile. The number of DNA fragments amplified by the 5 primes varied between 6 (primer OPA-11) and 10 (primer OPA-05) fragments. The cluster analysis phenogram of DNA fragments derived by Primer OPA-01 was the most reproducible one (Figure 4). In this phenogram, isolates F2, F5, F9, F14 were grouped in one cluster, referring to the close relationships between them. Interestingly, they also share the same mycelial compatibility group MCG4. Isolates F3, F6, F12 had quite similar DNA profiles with genetic similarity more than 94%, being members of MCG3. The isolates F8 and F7 were clustered in one cluster (MCG5) at genetic similarity of 93.29% (Figure 5). The isolate F4 separated in one cluster with F13 and F15. This isolate with F16 represents a separate MCG (G1). The loci generated by this primer were partially associated with the mycelial compatibility groups.

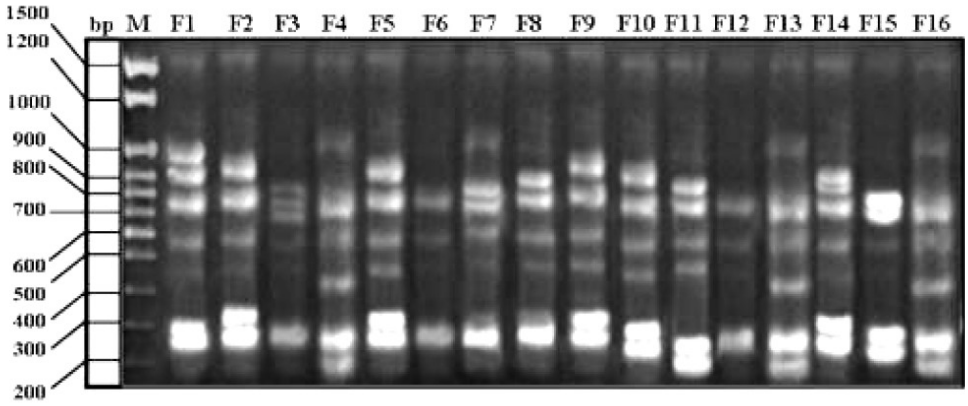

Figure 4 : Representative RAPD profile of *Fusarium oxysporum* isolates with, primer OPA-01.

DISCUSSION

The present study showed that all tested isolates of *F. oxysporum* were pathogenic to cotton cultivar (Giza 89). However, the pathogencity level between the isolates was entirely different, with regards to analysis of pre- and post infection stages and the resulting plant-fungus interactions. The variation in pathogenecity level between the different isolates might be due to some pathogenesis-related factors, such as hydrophobicity or attachment of germ line and changes in fungal metabolism, the factors that were reported to be the key elements in triggering of *F. oxysporum* pathogensis[49-52].

Present study indicated that there was heterogeneity among isolates of *F. oxysporum* obtained from different governorates in Egypt. Only 5 MCGs were obtained among *F. oxysporum* collected from 15 governorates in Egypt. This result confirmed the polyphyletic origin of *F. oxysporium* proposed by Skovgaard *et al*.[53]. It could be interpreted to mean that there are quantitative difference in compatibility associated with multiple loci as reported by Liu and Milgroom[54], or due to epistatic interaction among individual loci controlling incompatibility as indicated in the study of Huber and Fulbright[55]. Actually, the interaction between different genotypes, individuals, and isolates either prevent hyphal anastomosis or result in cell death following

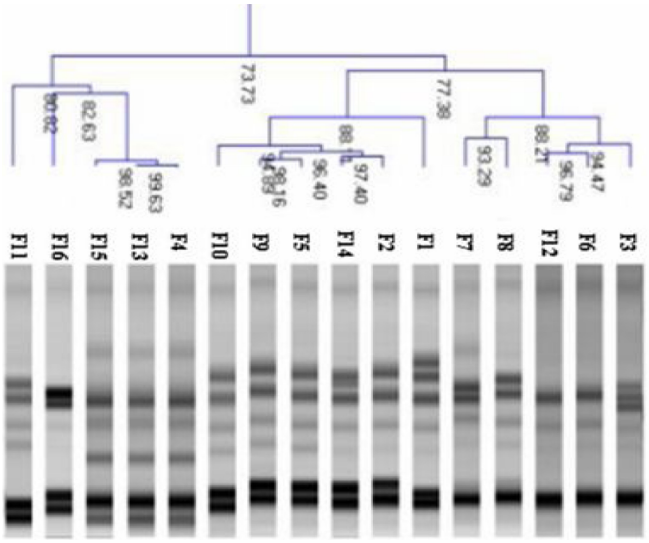

Figure 5 : Digitized patterns and dendrogram derived from RAPD profile analysis of *Fusarium oxysporum* isolates with primer OPA-01

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hyphal anastomosis[56]. Other factors may also interfere with compatibility, such the presence of dsRNA, which has been observed to confound compatible reaction[57-61].

F. oxysporum showed that the electrophoretic patterns of the studied isolates had 100% polymorphism. The cellular proteins grouped within a relatively wide molecular weight, ranged between 10 and 75 KDa. The wide variation in the electrophoretic patterns in the soluble proteins, as quantitative referring to the number of bands, as qualitative varying in their respective positions:[1] reflects the physiological state of the isolate rather than the morphological structure,[2] gives an exciting insights into the complex interactions that govern development in higher eukaryotic organisms, and[3] indicates that the similarity in the DNA sequences between the isolates is far less than 70%, the similarity limit in DNA sequences which reflected variation in protein profiles, as reported by Boriollo *et al*.[62].

The most pathogenic isolates were characterized with protein band with molecular weight of 60 KDa. This band was also present in F9, one of the least pathogenic isolate. The question mark is that "Is the 60 KDa in F9 the same band in the most pathogenic isolates?". If this band is an associated marker with pathogenicity, as we expected, the two protein subunits (the one present in F9 and that present in the most pathogenic isolates had the same molecular weight and different amino acid sequences). This means that they are coded from different genes and have different functions.

The level of genetic distance derived from cluster analysis of protein data was very close to that obtained by Aly *et al*.[63]. It ranged between 65 to 92% in the present study and between 62 to 97% in Aly *et al*.[63] study. This slight variation between Aly *et al*.[63] data and ours could be attributed to the variation in the temperature conditions, agitation, time of incubation, unfavorable environments and geographical localities.

The correlation between the virulence on one hand and the number of higher molecular weights bands[64-68] and the excess accumulation of the cellular proteins in the virulent isolates of some pathogenic bacteria[69-75] on the other hand was not observed in the present study. This means that such relation is species specific phenomenon and not a universal rule.

The polymorphisms observed for RAPD markers revealed a high degree of genetic diversity in *Fusarium oxysporum* isolates collected from different governorates in Egypt. This was consistent with the considerable genetic variation among the isolates of forma speciales of *Fusarium oxysporum* reported by Zamani *et al*.[76], Singh *et al*.[77] and Mostafa *et al*.[78]. This suggested that the polyphyletic origin of *F. oxysporum* is formed from a number of formae spciales. The considerable variation among the isolates of *F. oxysporum* is likely attributable to coevolution with a heterogeneous host population. Cotton is highly outcrossed and showed considerable genetic variability, providing ample opportunity for genetic evolution of the pathogen population.

The results from RAPD analysis and MCG analysis were corroborative (in terms of genetic diversity and not among them) and suggested a considerable genetic diversity among isolates *Fusarium oxysporum.* The loci generated by primer 1 were partially associated with the mycelial compatibility groups. However, some of MCGs were found within each RAPD group, indicating that although RAPD analysis and MCG analysis may both be good indicators of genetic variability within formae speciales of *Fusarium oxysporum*, the same grouping of isolates may not always be achieved with the two methods of analysis.

CONCLUSION

In conclusion, the pathogenic capabilities of the studied isolates were largely different, as pre-damping off ranged from 20 to 80%. The isolates were separated into 5 compatibility groups, with genetic distance ranged from 0.08 to 0.45. Group4 contained most of the studied isolates; the isolates with different pathogenecity. The whole cell protein bands of the studied isolates were polymorphic and the most pathogenic isolates were characterized with protein bands with molecular bands 60 Kda; the band that was also present in the low pathogenecity isolate F9. It was suggested that this band had a different genetic control and amino acids sequence in the isolates with high pathogenecity and isolate F9. The correlation between the virulence, and the number and intensity of higher molecular weights bands of the whole cell proteins in the virulent isolates is species specific phenomenon and not a universal rule as previously reported. The profile of the amplified bands by 5 primers gave an extensive variation between the tested isolates. Loci generated by primer 1 were

partially associated with the mycelial compatibility groups. We have the prospect to carry out this study on more isolates, collected from different macro-environments to derive a reliable conclusion on the relation between the pathogenicity and the macro-environments. The other prospect we are intend to do in future making an extensive study on assessing the diversity at molecular level, using more liable molecular techniques such as SSR, AFLP and isozymes.

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