ISSN: 2320-1983 Print ISSN: 2320-1991 Online Volume 2 Issue 4

Applied Cell Biology

Regular Paper

ACB, 2(4), 2013 [156-165]

Genetic diversity among some isolates of *Fusarium oxysporum* f. sp. *lycopersici* from tomato plants in Egypt

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Received: 15th October, 2013 ; Accepted: 19th November, 2013

ABSTRACT

A combined study of pathogenicity, mycelial compatibility, chemotaxonomic and molecular analyses was conducted to reveal the genetic variability in isolates of *Fusarium oxysporum* f. sp. *lycopersici* collected from tomato plants (stems and roots) in Egypt. The results derived from these techniques were corroborative and suggested a considerable genetic diversity among isolates. The extent of diversity, however, differed between analysis methods. The disease severity caused by the studied isolates showed a wide range of variation ranged from 42.2 % to 73.3 % on shoot system and from 31.1% to 62.2 on root system. The cluster analysis of mycelial compatibility data indicated no relationship between mycelial compatibility groups and virulence. The high percentage of polymorphism at both chemotaxonomic and molecular levels suggested a complex evolution pattern and confirmed the polyphyletic origin of *Fusarium oxysporium* f. sp. *lycopersici*. In addition, the partial association between the loci generated by one of the used primers and mycelial compatibility groups suggested that some of the latter were clonally derived. Intraspecific hybridization phenomenon was also discussed.

KEYWORDS

F. oxysporium f. sp. *Lycopersici*;
Tomato plants;
Pathogenicity;
Mycelial compatibility groups (MCGs);
Whole cell proteins;
RAPD analysis.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important Solanaceous vegetable crops allover the world under both outdoor and indoor conditions. It is subjected to attack by numerous soilborne pathogens. One of these devastated soil borne pathogens is *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) which attacks tomato seedlings in the nurseries and/or in the open fields after transplanting[1-4]. The infection of tomato plants by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) was recorded for the first time in Egypt by Fahmi[5]. This soil born pathogen causes drooping and downward curvature of the oldest leaves, usually followed by wilting and death of tomato plants.

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

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strains. These mechanisms are known as conidial anastomosis and hyphal anastomoses. In conidial anastomosis, anastomosis tubes are formed between conidia within acervuli. This mechanism was observed during *C. lindemuthianum* conidiogenesis[6-8]. 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"[9-12]. "Haploid nuclei in the heterokaryotic mycelium fuse together and form diploid nuclei. Through successive processes involving chromosome non-disjunction, the latter give rise to paternal haploids or recombinants"[9,13]. 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[10]. The *het* loci behave as if they were part of a recognition system that enables individuals to identify each other and to differentiate themselves from each other. The *het* loci can delimit the pathotypes of asexual, phytopathogenic fungi, as it occurs in the genus *Fusarium*[14-16].

Biochemical and molecular markers are being increasingly used to characterize fungal plant pathogen populations, including *Fusarium oxysporum* f. sp. *lycopersici*[17]. They are often versatile and highly informative tools for fungal pathogen identification at subspecific taxa, *i.e.*, *formae speciales* or physiologic races and diagnosis[18-21] and for population-genetic studies[22]. They can be used to evaluate levels of genetic diversity, systematic relationships and as an adjunct to morphological criteria in taxonomy[23-27].

Our objectives in this study were to analyze the diversity and genetic relationships of *F. oxysporum* f. sp. *lycopersici* isolates collected from cotton fields in a number of districts in Egypt using pathogenicity, mycelial compatibility, chemotaxonomic and molecular analyses

MATERIALS AND METHODS

Fusarium oxysporium f. sp. *lycopersici* (Sacc.) isolates were isolated from infected tissues of 20 stems and roots of tomato plants collected from fields in each of district (TABLE 1). The stems and roots were washed in tap water for 1 min, and cut into small pieces, 3-5 mm thickness. The pieces were surface sterilized by soaking in 70 % ethanol for 30 s, 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)[28]. The plates were incubated at 25 °C for 7 days. Once colonies of *Fusarium oxysporium* f. sp. *lycopersici* were established, they were transferred to slants of 1/4 strength PDA made for maintenance. All isolates were maintained over periods of 3 months on slants at 5 °C in a refrigerator for more frequent use.

TABLE 1 : Code, source and disease severity percent of the tested isolates of *Fusarium oxysporum* f. sp. *lycopersici* on shoot and root systems of tomato plants, cultivar (Castle Rock), after 45 days of inoculation.

Code of Isolates	Source of Isolate	Disease Severity %	
		Shoot System	Root System
501	Kafr el Shiekh (Balteem)	57.7	44.4
502	Kafr el Shiekh (Kafr el Shiekh)	42.2	31.1
503	North Sinai (El-Aresh)	66.6	53.3
504	El-Fayum (Abshway)	53.3	40.0
505	El-Sharkia (El-Sallhia)	55.5	42.2
506	El-Kalyubia (Tokh)	62.2	48.8
507	Beni Suef (Beba)	64.4	53.3
508a	El-Beheira (Nobaria)	48.8	37.7
508b	El-Beheira (Tahreer)	60.0	51.1
509	El-Gharbia (Kotor)	60.0	46.6
510	Ismailia (Abo- Soyer)	73.3	62.2
511	El-Menufia (Ashmon)	46.6	35.5
512	Sohag (Margha)	55.5	42.2
	Control	0.00	0.00

Identification

The isolated *Fusarium oxysporium* f. sp. *lycopersici* (Sacc.) from tomato was identified according to the descriptions of *Fusarium* by Gilman[29], Booth[30] and Barnett and Hunter[31] and confirmed by the identification unit in Mycological Research and Disease Survey Department, Agricultural Research Center, Giza, Egypt.

Pathogenicity test

Thirteen isolates of *Fusarium oxysporium* f. sp. *lycopersici* (Sacc.) were tested under the greenhouse conditions for their pathogenic potentialities on suscep-

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tible tomato cultivar (Castle rock), obtained from the Agriculture Research Center, Giza, Egypt.

Substrate for growth of the tested isolates of *Fusarium* was prepared in 500 mL glass bottles containing 100 g of sorghum grains, 50 g sand and 80 mL of tap water[19]. Contents of each bottle were autoclaved for 30 min; isolate inoculum, taken from one week old culture grown on potato dextrose agar (1/4 strength 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 tomato. Seeds of tomato were surface sterilized by submersion into 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[32,33]. 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 10 g/kg of soil. The prevailing temperature during pathogenicity tests was 30 ± 2 °C and seedlings were watered when necessary.

The percentage of disease severity in tomato was determined on the basis of leaf chlorosis and xylem discoloration according to the scale proposed by Woltz and Arthur[34].

Vegetative compatibility

Vegetative compatibility was determined as described earlier[35]. 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[36-40]. 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 25 °C 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 –80 °C for further use. Two grams of the frozen mycelia were ground with a mortar in liquid nitrogen to a fine powder. 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[41,42] and stained with silver nitrate[43]. To ensure that data of the whole cell proteins analysis was consistent and convinced, the whole cell proteins analysis was repeated three times.

Multilocus-enzymes extraction

The extraction of enzymes from fungi was carried out in the same way as previously mentioned for whole cell proteins with some modifications. The fine powder of the frozen mycelia was brought to 200µl with extraction buffer (50 mM Tris-HCl (pH 6.8), 10 % w/v glycerol, 0.1 % ascorbic acid, 0.1 w/v cycteine hydrochloride). The mixture was centrifuged at 10,000 rpm for 30min. Protein content was measured according to Bradford[44], adjusted to 3mg/ml per sample.

Multilocus-enzymes electrophoresis

Isozymes electrophoresis was performed in vertical polyacrylamide gels with a discontinous buffer system as described by Aly *et al.* (19). The following enzyme systems were screened: esterase (EST, 3.1.1.1), malate dehydrogenase (MDH, 1.1.1.37), and peroxidase (POX, EC 1.11.1.7). The staining protocols used in this study were that described by Gall *et al.*[45].

Genomic DNA extraction

Three to four mycelial plugs (each 4 mm in diameter) from PDA cultures were transferred to flasks con-

taining 150 mL of potato dextrose broth, which were incubated at 25 °C ± 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 % and 97 %, and the ratio between 1.7-1.8. Concentration was adjusted at 6 ng/µL for each sample before amplification.

RAPD analysis

Genomic DNA from *Fusarium oxysporum* was amplified by the RAPD using 15 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 4 min, 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, isozymes and RAPD analyses for each isolate were compared on the basis of the presence (1) versus absence (0) of vegetative compatibility reaction, protein band, isozyme 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[46]. 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

Tomato plants artificially infected with the studied isolates showed wilt symptoms. The first symptom appeared on the infected plants was yellowing of the foliage; began with the lower leaves and worked upward. Infected leaves later showed downward curling, followed by browning and drying. Wilting becomes progressively worse until the entire vine was permanently wilted. Vascular browning could be seen in infected stems and large leaf petioles. Finally, infected plants and their root systems were stunted. The tested isolates did not posses the same level of disease severity (TABLE 1). The disease severities of the shoot and root systems percentages ranging 42.2% to 73.3% and 31.1% to 62.2% respectively. The highest disease severity was reported for the isolates 510, followed by isolates 503 and 507 respectively. The corresponding values of disease severity percentages for the former isolates on shoots were on average 73.3%, 66.6% and 64.4% and on roots were 62.2%, 53.3% and 53.3%, respectively. Meantime, isolate 502 caused the lowest percentage of disease severity either on shoot or on root system, being 42.2% and 31.1%, respectively. Other isolates had intermediate disease severity (TABLE 1). The variation in disease severity between different isolates was significant, as indicated by LSD values.

The thirteen isolates of *Fusarium oxysporium* f. sp. *lycopersici* were subjected to study of vegetative compatibility. The results of vegetative compatibility indicated that all isolates were self-compatible. The ap-

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plication of UPGMA clustering produced 5 mycelial compatibility groups (MCGs) (Figure 1), with genetic distance ranging from 0.08 to 0.54 genetic similarity. Each mycelial compatibility group often includes isolates with different disease severities. Some of these isolates were highly pathogenic, whereas others exhibited a low pathogenicity. Isolate with the lowest pathogenic potential (508A) grouped with the highest pathogenic ones (510) at genetic distance of 0.23. On the other hand, isolates with the highest pathogenic potential (510, 507 and 503) grouped in two distanced groups (G1 and G4), at genetic distance of 0.54.

The whole cell proteins of the tested isolates of

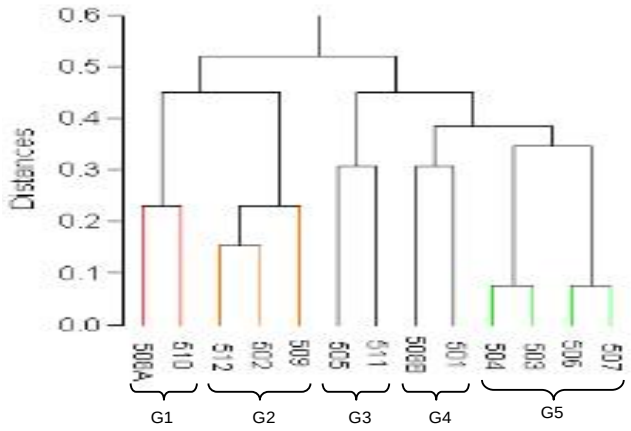

Figure 1 : Cluster analysis derived from vegetative compatibility data of *Fusarium oxysporum* f. sp. *lycopersici.*

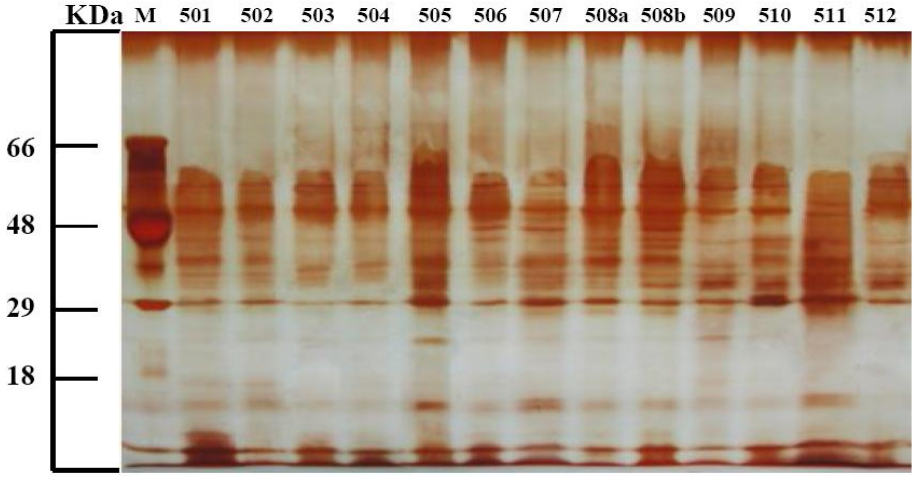

Figure 2 : SDS-polyacrylamid gel electrophoresis profile, stained with silver nitrate, of 13 *Fusarium oxysporum* f. sp. *lycopersici* isolates

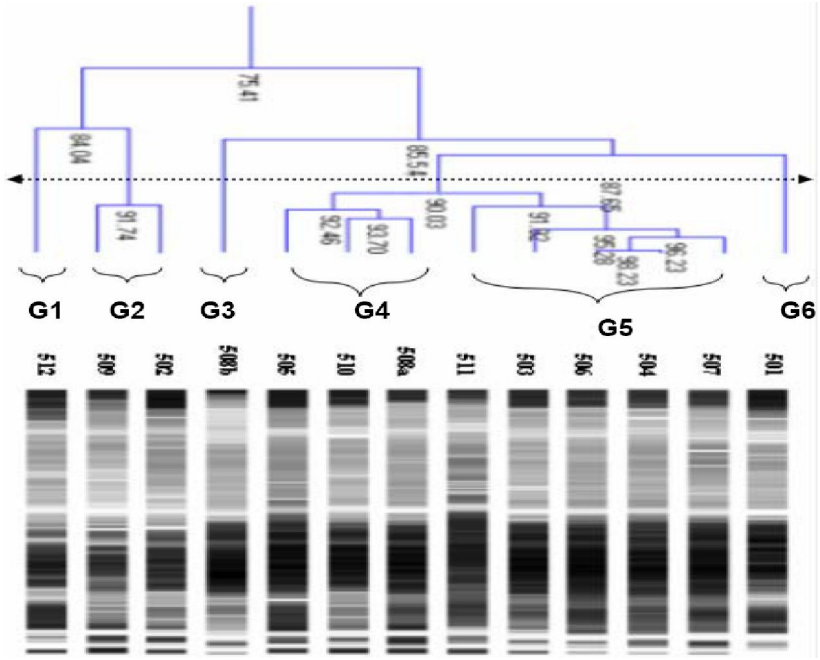

Figure 3 : Digitized patterns and dendrogram derived from protein profile analysis of 13 *Fusarium oxysporum* f. sp. *lycopersici* isolates using SDS-polyacrylamid gel electrophoresis, stained with silver nitrate.

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Fusarium oxysporum f. sp. *lycopersici* were electrophoreticaly analyzed on SDS/PAGE (Figure 2), showing a great number of polymorphic bands with molecular weights ranging from 14 KDa to 70 KDa. Generally, there was no definite relation between the number or the intensity of whole cell proteins bands and pathogenicity. The cluster analysis based on data of whole cell proteins data separated the isolates of *Fusarium oxysporum* f. sp. *lycopersici* into five clusters (Figure 3). Often the same group may includes a mixture of isolates with different disease severity.

The activity and resolution of three enzyme-staining procedures for *Fusarium oxysporum* f. sp. *Lycopersici*. were tested. Poor resolution was observed for peroxidase and malate dehydrogenase (data not shown), while esterase enzyme showed strong activity and acceptable resolution. The data of esterase enzyme gave 2 zones of activities (Figure 4); the anodal zone and the cathodal zone. The cathodal zone contained one band, with different intensity, whereas the anodal zone included one band with different mobility, thickness and intensity, except 507 which showed two bands. The cluster analysis based on esterase analysis data produced two major groups; one group included the isolate 502 (the lowest pathogenic isolate) and the second group divided into four clusters joined at genetic similarity of 88.52% (Figure 5). The isolates of each cluster were not homogenous as to the pathogenicity.

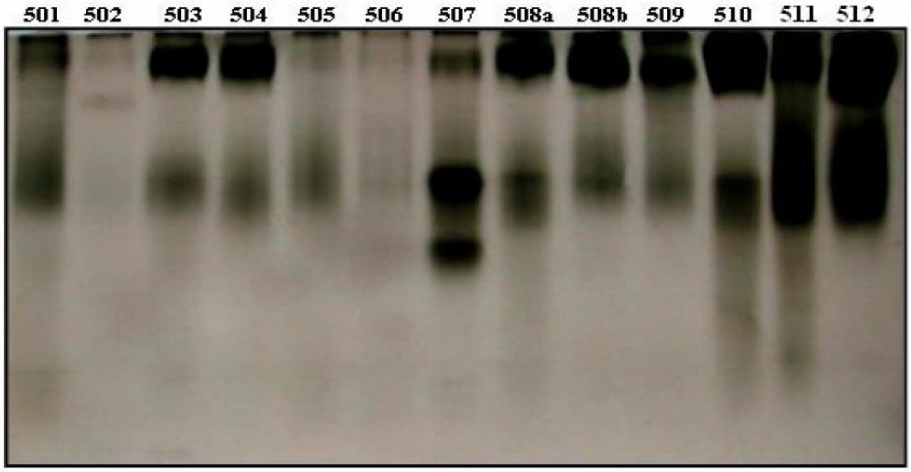

Figure 4 : Esterase profile analysis of 13 *Fusarium oxysporum* f. sp. *lycopersici* isolates, using native polyacrylamid gel electrophoresis.

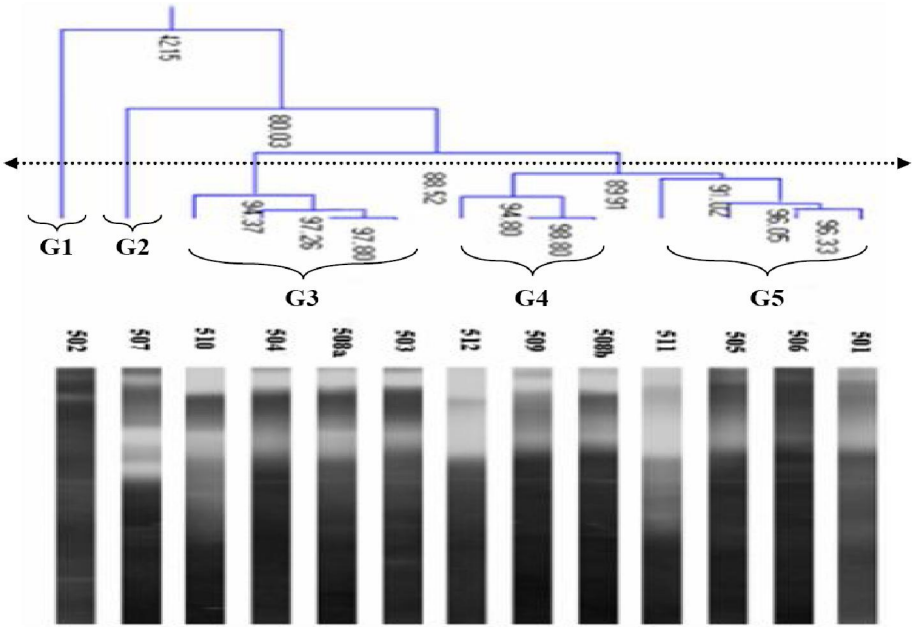

Figure 5 : Digitized patterns and dendogram derived from esterase profile analysis of 13 *Fusarium oxysporum* f. sp. *lycopersici* isolates, using native polyacrylamid gel electrophoresis.

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Genomic DNA isolated from thirteen isolates of *F. oxysporum* f. sp. *lycopersici* was subjected to RAPD-PCR analysis with fifteen random decamer primers. In the preliminary experiments, seven out of the fifteen primers tested produced distinct and reproducible band profiles. However, five only generated polymorphic bands in all isolates. Three of the five primers were used for a comparative analysis of the thirteen isolates of *F. oxysporum* f. sp. *lycopersici*. These primers were OPA-06 (52 -GGTGCGGGAA-32), OPA-8 (52 -GTTTCGCTCC-32) and OPA-14 (52 -CCCGTCAGCA-32) (Figure 6). Amplified fragments were ranged from 200 bp to 1.7 kb. The DNA fragments generated by primer 8 proved that isolate 501 could be a hybrid of isolates 502 and 505 (Figure 6B). The number of DNA fragments, amplified and scored per isolate for individual primer, ranged from 2 to 10. These data show that RAPD is a convenient method for distinguishing the isolates of *F. oxysporum* f. sp. *lycopersici* and also reveal a high degree of genetic variation among the isolates. The cluster analysis based on DNA fragments generated by Primers 6,8,14 gave four clusters, G1-G4 (Figure 7). The isolates with high disease severity were collected in one cluster (G4) and the isolates with low disease severity grouped in cluster (G3).

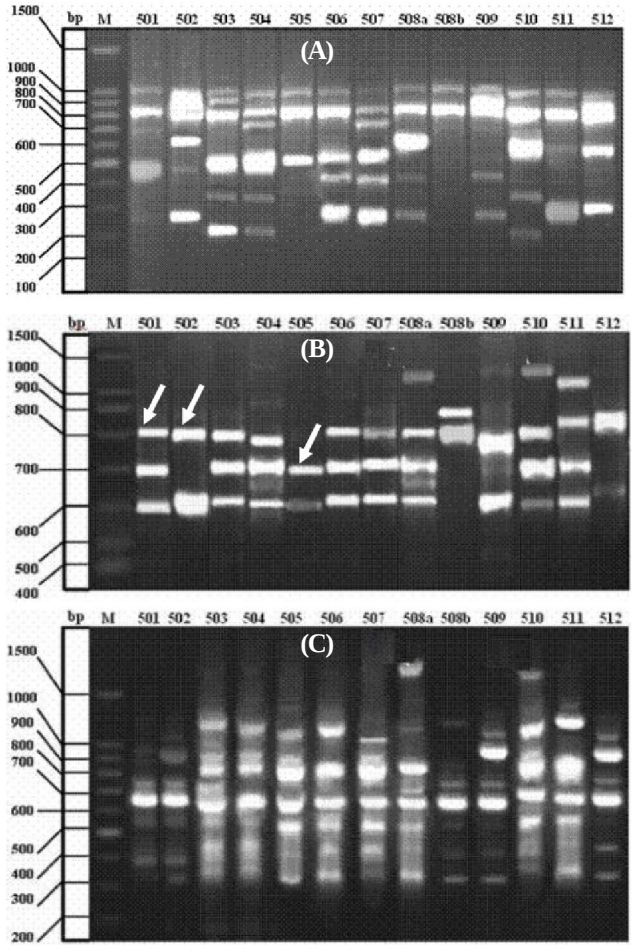

Figure 6 : RAPD patterns on 1.2% agarose gel of amplified fragment generated from 13 isolates of *Fusarium oxysporum* f. sp. *Lycopersici* with random primers (A) OPA-06 (52 -GGTGCGGGAA-32), (B) OPA-8 52 -GTTTCGCTCC-32) and (C) OPA- 14 (52 -CCCGTCAGCA-32)..

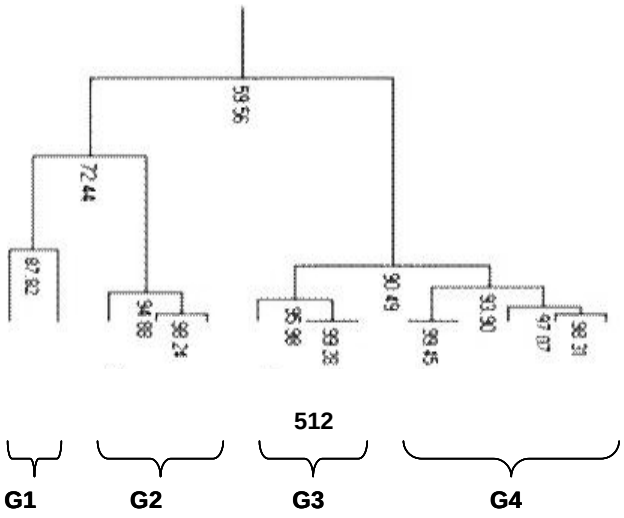

Figure 7 : Dendogram derived from RAPD profile analysis of 13 *Fusarium oxysporum* f. sp. *lycopersici* isolates, using random primers (A) OPA-06 (52 -GGTGCGGGAA-32), (B) OPA-8 52 -GTTTCGCTCC-32) and (C) OPA- 14 (52 -CCCGTCAGCA-32).

DISCUSSION

Genetic characterization of *F. oxysporum* f. sp. *lycopersici* is essential for the efficient management of Fusarium wilt through use of resistant cultivars in tomato growing areas. This study demonstrated that pathogencity, mycelial compatibility, cellular proteins, isozyme (esterase) polymorphisms and RAPD-PCR provided valuable information as to the degree of variability and level of genetic relatedness among the tested isolates of *F. oxysporum* f. sp. *lycopersici*. Understanding of occurrence, distribution and genetic relatedness of such pathogenic variants is a prerequisite for developing effective and efficient disease management; but only a few surveys have been conducted on this devastating pathogen in Egypt.

The data of pathogenecity test showed that all the tested isolates of *F. oxysporum* f. sp. *lycopersici* were pathogenic to tomato plants cultivar (Castle Rock). The level of disease severity, however, varied from isolate

to another. The variation in disease severity, may be attributed to the variation in the virulence factors, the secretion enzymes of the pathogen[47] or may be due to the virulence factors that promote fungal colonization, e.g. the adherence to host cells.

Five MCGs were obtained among *F. oxysporum* f. sp. *lycopersici* isolates, indicating a heterogeneity among isolates of *F. oxysporum* f. sp. *Lycopersici*. This data could be interpreted to mean that there are quantitative difference in compatibility associated with multiple loci[48], or an epistatic interaction among individual loci controlling incompatibility[49].

The electrophoretic patterns of the studied *F. oxysporum* f. sp. *Lycopersici* isolates had a high percentage of polymorphism, about 90%. The whole cell proteins grouped within a relatively wide molecular weight, ranging between 10 kDa and 70 kDa. The wide variation in the electrophoretic patterns in the whole cell 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[50].

The electrophoretic patterns of the studied isolates showed no pronounced variation in the number and intensity of the major protein bands between high and low pathogenic isolates. This finding ruled out the concept that virulence is related to the increase in the number of major protein bands[51-55] and the accumulation in protein bands in the highest pathogenic isolates is due to the expression of the *vrl*. locus[56-61]. We suggested that variation in the whole cell proteins could be species specific or could be due the environmental stresses which the isolates subjected to in their natural habitat.

The polymorphisms observed for RAPD markers revealed a high degree of genetic diversity in *F. oxysporum* f. sp. *Lycopersici* isolates collected from different districts in Egypt. In Egypt there is scarce studies on the genetic diversity of *F. oxysporum* f. sp. *Lycopersici* based on molecular data. Even, if such study is present, it focuses on the assessment of the genetic relationship between *Fusarium* species, including *F. oxysporum* f. sp. *Lycopersici*[17]. The considerable variation among the isolates of *F. oxysporum* f. sp. *Lycopersici* is likely attributable to coevolution with a heterogeneous host population. Tomato is highly outcrossed and showed considerable genetic variability, providing ample opportunity for genetic evolution of the pathogen population.

OPA-08 gave some loci (DNA fragments) which proved that some isolates could be a hybrid between other two isolates. For example, isolate 501 could be a hybrid between the two isolates 502 and 505. This phenomenon was reported in many pathogens[62-66]. However, as far is know, our data is the first evidence revealing recombination between two genes in *Fusarium oxysporum* f. sp. *Lycopersici*. Although, it was suspected that there was a hybrid between the studied isolates, the present data was not sufficient to prove the recombinant status of these isolates. It will be necessary to include more molecular data to obtain an accurate reflection of the presence of hybrids in natural populations. This data showed how the trade and the movement of human being enhanced the possibilities of hybridization between allopatric and sympatric taxa that are partially reproductively isolated.

The cluster analysis of the RAPD data proved that Egyptian isolates were originated from four different genetically colons. The isolates derived from each colon had a close degree of pathogenicity and need to be managed differently. Interestingly, the lowest pathogenic isolates and the highest pathogenic isolates were separated into two different groups (G3 and G4), based on RAPD data generated by the three primers OPA-06, OPA-08 and OPA-14.

In conclusion, isolates of *Fusarium oxysporum* f. sp. *lycopersici* were varied and had existing high level of genetic variation. These observations could be helpful for further breeding studies of tomato cultivars resistant to this destructive pathogen.

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