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ORIGNAL PAPER

Fusarium oxysporum Isolates Collected from the same Geographical Zone Exhibited Variations in Disease Severity and Diversity in Morphological and Molecular Characters

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ABSTRACT

Thirty *Fusarium oxysporum* f. sp. *cucumerinum* (FOC) isolates were collected from Kafr El-Sheikh governorate, Egypt to study their pathogenic variability and genetic diversity. All isolates caused the typical symptoms of wilting with different severity degrees and reduced cucumber growth parameters and crop productivity under greenhouse conditions. We have classified the isolates to different groups based on pathogenicity, morphological characters, disease quantum degrees and genetic variation. The polymorphisms obtained from RAPD and protein profiles revealed a high degree of genetic diversity and their cluster analysis was matched with the results obtained from the pathogenicity. Previously it was believed that the different collection regions of the same pathogen isolates were the cause for their pathological and morphological differences, and there was no genetic variation can be occurring between isolates as long as they were collected from the same place. In our results, the genetic diversity of FOC isolates proves that the genetic variation of fungus isolates can occur in the same geographical location.

Key words: *Cucumis sativus*, *Fusarium oxysporum* f. sp. *cucumerinum*, Molecular diversity, Protein analysis, RAPD, Vascular wilt.

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INTRODUCTION

Cucumber (*Cucumis sativus* L.) is an important economical vegetable crop all over the world. It ranks third of the important vegetable crops after tomato and onion in terms of total global food production. China is the main country of cucumber cultivation followed by Iran, while Egypt comes in 13th in productivity (FAOSTAT database, 2018). Cucumber is a source of many vitamins, folic acid, pantothenic acid, antioxidants, minerals and fibers (Adinde *et al*., 2016). Cucumber is considered a precious catch of many pathogens such as fungi, bacteria and viruses. Undoubtedly, fungi are the most destructive pathogens on cucumber. Fusarium wilt caused by *Fusarium oxysporum* (Schlechtend.:Fr.) f. sp. *cucumerinum* (Owen) Snyder & Hansen (FOC), comes at the head of fungal pathogens list as the most aggressive soil-borne disease of cucumber (Armstrong and Armstrong, 1978), it causes a very harmful and severe economic damage on cucumber crop in various regions around the world, such as Canada, Spain, China, Japan, Egypt, England, Greece, Thailand, South Africa, Germany, France and Australia (Booth, 1971; Vakalounakis, 1988; Martínez, *et al*., 2003; Vakalounakis *et al*., 2004). *Fusarium oxysporum* has a high ability to infect plants at any time of growing stage by attacking directly to the roots or through wounds. It penetrates root cortex and inwards to the xylem vessels forming a dense mass of mycelium (Wang *et al*., 2015). As soon as the microconidia are formed inside the plant, it will be directed upward in the plant vascular system with the help of sap stream and move up from the root to shoot system. Both mycelium and spores together are involved in the blockage of the vessels and obstruct the transmission of nutrients

to the rest of the plant and the water loss amount by transpiration becomes much greater than the ability of the roots to absorb water, resulting in the closure of stomata. Then the plant begins to enter the wilting phase, which inevitably ends with plant death. This in turn, affects the quantity and quality of the crop yield value under greenhouse and open field conditions (Manikandan *et al*., 2018). The risk of the Fusarium wilt is not limited to this damage, but the real danger lies in its ability to remain in the soil as chlamydospores, which can survive for many years as a strong source of new infection as epidemic disease and retain the vitality to invade cucumber tissues in the coming years. Disease symptoms of *Fusarium*, appear typically on the old leaves and progress to the younger foliage as yellowing of leaves, followed with necrotic lesions, then foliar wilting, vascular discoloration in the root and stem and eventually plant death. Cucumber fruits become small-sized and low in quality (Vakalounakis, 1988; Vakalounakis *et al*., 2004). Differences in morphological traits between fungus isolates are used as an effective means for distinguishing between them such as texture, shape, size and pigmentation of colonies, growth rate of mycelium, shape and size of spores. With scientific progress, reliance on morphological studies to distinguish between isolates has become imprecise. The uses of molecular techniques become more efficient and exact to differentiate fungal species and strains (Mezzomo *et al.,* 2018). Biochemical and molecular markers have assessed the genetic diversity and phylogenetic relationships between different isolates to distinguish and explain their variation in pathogenicity (Welsh and McClelland, 1990; Williams *et al.,* 1990). Pathogenicity, protein electrophoresis and RAPD were used to characterize between isolates of *Fusarium solani* f. sp. *cucurbitae* (Crowhurst *et al.*, 1991), *Fusarium oxysporum* f. sp. *pisi* (Grajal-Martin *et al.,* 1993) and *Fusarium oxysporum* f. sp. *cucumerinum* (Vakalounakis and Fragkiadakis, 1999). In the present study, attempts were made to identify *F. oxysporum* f. sp. *cucumerinum* isolated from wilted cucumber and assessment the pathogenic ability and the morphological characterization of the isolates. We utilized proteins pattern and polymerase chain reaction to differentiate the *Fusarium* isolates and study their genetic diversity. Our main objective is to determine the genetic diversity of some FOC isolates collected from the same area and correlating this with their ability to cause cucumber wilt.

MATERIALS AND METHODS

1- Isolation and identification of the causal organism:

A total of 30 isolates of fungi were isolated from naturally infected cucumber plants. Plants surveyed from different hotspots of five fields close to each other in two locations adjacent together (Qallin and Kafr El-Sheikh) in Kafr El-Sheikh governorate, Egypt (Figure 1).

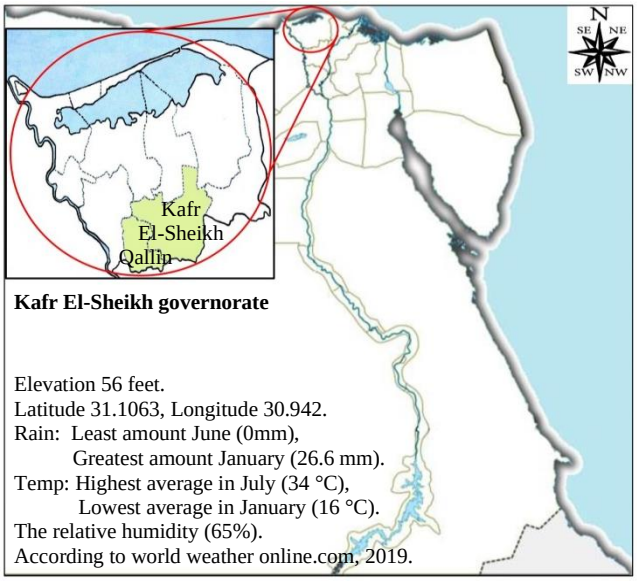

Figure (1): Map showing geographical surveying location of *Fusarium oxysporum* f. sp. *cucumerinum* isolates in Kafr El-Sheikh governorate, Egypt (shaded with green color).

The fields were chosen to collect the infected plants based on the appearance of the typical wilt symptoms with differences in the symptom's stages from the yellowing of leaves to plant death. Roots of the diseased plants were washed with tap water to remove soil particles, infected tissues were surface disinfected in sodium hypochlorite (3.0 %) for 3 minutes, washed with sterilized distilled water for several times, dried using sterilized filter paper and transferred into Petri dishes containing Potato Dextrose Agar medium (PDA). Plates were incubated at 27°C for 24 to 48 hours. The pure cultures were obtained by the hyphal tip technique (Hawker, 1956), examined microscopically, and identified according to Booth (1971) and Leslie and Summerell (2006). Identification of pathogen was confirmed in

Mycological Research and Disease Survey Department, Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt. The different *Fusarium* isolates were coded with FOC-1 to 30, maintained on PDA slants and kept at 4°C for further studies.

2- Host range test:

The 30 isolates of *F. oxysporum* were tested against six plant species belong to four plant families: Cucurbitaceae (cucumber, *Cucumis sativus* cv Beit Alpha; watermelon, *Citrullus lanatus* cv Giza 1 and cantaloup, *Cucumis melo* cv Ananas). Bean *Phaseolus vulgaris* cv Valentino from Fabaceae; tomato, *Lycopersicon esculentum* cv G.S. from Solanaceae and cabbage *Brassica oleracea var. capitat*a cv Balady from Brassicaceae. Three seeds from (watermelon, cantaloupe and bean) and three seedlings from (cucumber, tomato and cabbage) were surface-sterilized and transplanted to 30 pots containing *Fusarium oxysporum* inoculum. Seeds and seedling planted in free inoculum soil served as controls. Infected plants were maintained under greenhouse conditions. Seedlings were watered as required to maintain normal growth. Disease incidence was measured 15 days after transplanting. Host range test was repeated three times for each fungal isolate.

3- Phenotypic Characterization of FOC:

Thirty FOC isolates were grown on PDA medium each alone to study their morphological characters. 5 mm disc was plugged from 7 days old pure culture and transferred to the center of sterilized Petri dish containing 20 ml of PDA. The plates were incubated at 27°C for 7 days. Experiment was repeated three times for three independent biological replicates each consisting of 3 plates. The mycelial growth, colony color, sporulation degree and growth type were recorded.

4- Pathogenicity test:

Seedlings of cucumber susceptible cultivar "Beit Alpha" were grown from surface-disinfected seeds and kept under greenhouse conditions at the Department of Vegetable Crops, Horticultural Research Institute, Dokki, Giza, Egypt, at 25 ± 2°C with 60-70% relative humidity for 30 days. Inoculum was prepared by growing isolates in autoclaved bottles each contained (100 g sorghum, 50 g sand and 80 ml water) at 27°C for 21 days (Abd-Elsalam *et al*., 2007), pots (30 cm diameter × 27 cm height) were sterilized using 5% formalin and left for 2 days to ensure complete formalin evaporation. Pathogen-free sandy loam soil was used for planting. Soil infestation was carried out by adding the previous inoculum to each pot at the rate of 3% of the soil weight. Sterilized sorghum grains at the same rate were used as a control. Pots were watered every 2 days for 2 weeks to ensure the establishment of the isolates in the soil, no fertilizers were applied.

After 30 days from sawing date three seedlings were transplanted to every pot. Five pots were used for each isolate. The pathogenicity test for all isolates was carried on the seedlings under greenhouse conditions of Vegetable Diseases Research Department, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt. Disease severity of infected plants was assessed after 15 and 30 days from transplanting using a scale rating from 0 to 5 based on the leaf wilt symptoms according to Liu *et al.* (1995) as follow:

0 = no symptoms; 1 = Plants up to 25% of leaves with symptoms; 2 = Plants with >25-50% of leaves with symptoms; 3 = Plants with >50-75% of leaves with symptoms; 4 = Plants with >75-100% of leaves with symptoms and 5 = Plants with complete death. Disease severity (%) was calculated using the following formula:

$$\text{Disease severity (\%)} = (\sum nr/5N) \times 100$$

Where:

n = number of plants in each category, r = rating category, N = total number of plants and 5 = highest rating category.

Based on disease severity percentages the aggressiveness of different isolates was categorized into 4 groups: slightly aggressive (1-25%), moderately aggressive (>25-50%), highly aggressive (>50-75%) and extremely aggressive (>75-100%) according to Šišić *et al*. (2018). The effect of FOC isolates on plant growth parameters: plant height (cm), fresh and dry weights for root and shoot (g), and average of plant product (kg) were recorded. All the tested parameters were measured after 30 days from transplanting, 3 plants were used as independent biological replicates.

5- Fungal material:

The purified FOC isolates were transferred each alone to 100 ml Potato dextrose Broth (PDB) and incubated at 27°C for 7 days. 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.

6- Genomic DNA extraction:

All isolates were crushed to a fine powder using mortar and pestle in liquid nitrogen. 50 mg were transferred in 2 ml tube and 600 µl of extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) were added along with 5µl RNAse as described by Raeder and Broda (1985), all tubes were incubated for 10 minutes at 60°C. 300 µl of 3 M sodium acetate, pH 5.2 were added, tubes were placed at -20°C for 10 minutes. Tubes were centrifuged at 13000 g for 5 minutes and the supernatant was transferred to another tube. An equal volume of isopropanol was added, all tubes were incubated for 10 minutes at room temperature and the precipitated DNA was pelleted by centrifugation at 13000 g for 5 minutes. Pellets were washed with 70% ethanol, air-dried and dissolved in 50 µl of TE buffer. The quantity and quality of the extracted DNA were estimated using Nanodrop1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

7- RAPD analysis:

Fungal DNA from each isolate was amplified by the RAPD using 15 random primers from Operon primer kit (Operon Technology Inc, USA), list of primers is available at the end of the article (Vakalounakis and Fragkiadakis, 1999; Vakalounakis *et al*. 2004; Najafiniya and Sharma, 2011). PCR amplifications were performed in a total volume of 20 µl containing 25 ng genomic DNA, 50 mM KCl, 2 mM $MgCl_2$, 10 Mm Tris/HCl (pH8.3), 400 µM dNTPs, 0.2 µM primer 100 ng and 0.5U of Taq DNA polymerase (PromegaCorp. Madison, WI) and carried out in PTC100 programmable thermocycler (MJResearch, Water Town, MA, USA). The PCR programme used was 94°C for 5 minute, 35 cycles for 1 minute at 94°C, 36°C for 1 minute and 72°C for 2 minutes as a final extension (Gupta, 2012). 10 µl of PCR reaction product were resolved on 2% agarose gel at 50 V, visualized by ethidium bromide stain (0.5 µg/ml), photographed using ChemiDoc™ MP Imaging System version 5.2. A 1kb DNA ladder (Thermo Fisher Scientific) was used as the molecular weight standard.

8 - Protein extraction:

Two grams of the frozen mycelia from each isolate were ground using liquid nitrogen to a fine powder. The total protein was extracted using the TCA/acetone method as described by Fernández and Jorrín Novo (2013) with some modifications. 1 ml of 10% (w/v) TCA/acetone was added to 100 mg of mycelial powder and mixed well by vortexing. Samples were sonicated 5 × 15 s (50 W, amplitude 60) at 4°C and were breaked on the ice at 1 minute. The tube filled with 10% (w/v) TCA/acetone, mixed well by vortexing and centrifuged at 16,000 g for 5 minutes (4°C). The pellet was washed with 0.1 M ammonium acetate in 80% (v/v) methanol and then with 80% (v/v) acetone, the tubes were air-dried at room temperature to remove residual acetone. 1.2 ml of 1:1 phenol (pH 8, SIGMA)/SDS buffer were added and mixed well by vortexing. The tubes were incubated for 5 minutes in ice, centrifuged at 16,000 g for 5 minutes. The upper phenol phase was transferred into a new 2 ml tube; 0.1 M ammonium acetate in 100% (v/v) methanol was added, mixed well and allowed the precipitation overnight at −20°C. The tubes were centrifuged at 16,000 g for 5 minutes (4°C). The pellet was washed with 100% methanol and with 80% (v/v) acetone, respectively, air-dried and resuspended in rehydration buffer [8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT] at 25°C. Protein concentration was determined by Bradford assay (Laemmli, 1970).

9- Electrophoretic analysis of proteins by SDS-PAGE:

The extracted proteins (150 µg) of all 30 isolates along with low range pre-stained marker (Bio-Rad) were separated on 1DE. Stacking gel of 4% acrylamide/bisacrylamide (30%/0.8%) and resolving gel of 12.5% acrylamide/bisacrylamide (30%/0.8%) were subjected to 50 V for 30 minutes and then at constant 70 V. Proteins were stained with CBB G-250. Gel images were scanned with ChemiDoc™ MP Imaging System with Image Lab™ Software version 5.2 (Bio-Rad, USA).

10- Data analysis of RAPD and Protein:

The data generated from RAPD analyses and total cell proteins for each isolate were compared based on measurement of band position along the lane, relative to the lane length which known as retardation factor (RF). Relative relatedness among isolates was determined using CLIQS v1.1 software. A dendrogram representing the phylogenetic relationship between the isolates was constructed from the matrix of dissimilarities by the unweighted pair-group method algorithm (UPGMA). DNA and protein experiments were repeated three times for three independent biological replicates.

11- Statistical analysis:

The data were statistically analyzed by ANOVA, using wasp software (Web Agriculture Stat Package). The values presented are the means of all measurements. Differences among the means were compared and determined by Duncan's multiple range tests using the least significant difference test at $p<0.05$ (Gomez and Gomez, 1984).

RESULTS

1- Isolation of the pathogen:

The 30 isolates obtained from cucumber plants, were identified as *Fusarium oxysporum* in Mycological Research and Disease Survey Department, Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt.

2- Host range test:

All 30 *F. oxysporum* isolates tested for host range showed the typical Fusarium wilt symptoms only on cucumber cv Beit Alpha. While no symptoms were visible on the other inoculated plant species which clearly demonstrated cucumber-specific infection (Table 1). The forma speciales concept in the *F. oxysporum* is restricted to those causing wilt of a specific host (Snyder and Toussoun, 1965; Correll, 1991; Dissanayake *et al*., 2009). Thus, the thirty *F. oxysporum* isolates were considered as *Fusarium oxysporum* f. sp. *cucumerinum.*

Table (1): Host range of *Fusarium oxysporum* isolates, isolated from cucumber, surveyed from 2 locations (Qallin & Kafr El-Sheikh) 15 days post-inoculation under greenhouse conditions.

Isolate code	Cucurbitaceae			Fabaceae	Solanaceae	Brassicaceae
	Cucumber	Watermelon	Cantaloup	Bean	Tomato	Cabbage
	Beit Alpha	Giza 1	Ananas	Valentino	G.S.	Balady
FOC-1	+	-	-	-	-	-
FOC-2	+	-	-	-	-	-
FOC-3	+	-	-	-	-	-
FOC-4	+	-	-	-	-	-
FOC-5	+	-	-	-	-	-
FOC-6	+	-	-	-	-	-
FOC-7	+	-	-	-	-	-
FOC-8	+	-	-	-	-	-
FOC-9	+	-	-	-	-	-
FOC-10	+	-	-	-	-	-
FOC-11	+	-	-	-	-	-
FOC-12	+	-	-	-	-	-
FOC-13	+	-	-	-	-	-
FOC-14	+	-	-	-	-	-
FOC-15	+	-	-	-	-	-
FOC-16	+	-	-	-	-	-
FOC-17	+	-	-	-	-	-
FOC-18	+	-	-	-	-	-
FOC-19	+	-	-	-	-	-
FOC-20	+	-	-	-	-	-
FOC-21	+	-	-	-	-	-
FOC-22	+	-	-	-	-	-
FOC-23	+	-	-	-	-	-
FOC-24	+	-	-	-	-	-
FOC-25	+	-	-	-	-	-
FOC-26	+	-	-	-	-	-
FOC-27	+	-	-	-	-	-
FOC-28	+	-	-	-	-	-
FOC-29	+	-	-	-	-	-
FOC-30	+	-	-	-	-	-

3- Phenotypic Characterization of *F. oxysporum* f. sp. *cucumerinum* Isolates:

Based on the phenotypic characterization we studied some of the characteristics to distinguish among FOC isolates, such as the colonies color, mycelial growth type, sporulation degree and the growth rate of different isolates, the results are recorded in Table 2. The colors of the colonies for all isolates were greatly varied and divided into four main groups (violet, pink, brown and white), each group was divided into subgroups depending on the varying shades of the pigments between colors *viz*., violet group contains violet (FOC-15), pale violet (FOC-2), white with violet (FOC-1 and 23), white with pale violet (FOC-5, 6, 8, 20 and 21), creamy with dark violet (FOC-10), creamy white with pale violet (FOC-3, 30), creamy white with dark violet (FOC-13, 17) and milky white with pale violet (FOC-29). The pink group contains pale pink (FOC-11), white with dark pink (FOC-24), creamy with pale pink (FOC-4), creamy white with pale pink (FOC-12, 16, 18 and 19) and milky white with dark pink (FOC-27). The brown group contains dark brown with white (FOC-7, 14, 22 and 28) and milky white with light brown (FOC-26). The white group contains creamy white (FOC-9) and milky white with light yellow (FOC-25). Furthermore, the study of mycelial growth pattern cleared 3 different patterns, fluffy (15 isolates), fluffy-smooth (9 isolates) and smooth (6 isolates). Based on sporulation degree, the isolates were divided into 4 categories, poor (6 isolates), medium (6 isolates), good (11 isolates) and high sporulation (7 isolates). Based on the growth rate measurements, there are 10 isolates had a slow growth rate, 8 isolates had medium growth rate and 12 isolates had fast growth rate (Figure 2, Table 2).

Table (2): Morphological characters of FOC isolates collected from 2 locations (Qallin & Kafr El-Sheikh) under *In vitro* conditions.

Isolate code	Colony color	Mycelial growth pattern	Sporulation degree	Growth rate
FOC-1	White with violet	Smooth	++++	Fast
FOC-2	Pale violet	Smooth	+	Slow
FOC-3	Creamy white with pale violet	Fluffy, smooth	+++	Medium
FOC-4	Creamy with pale pink	Smooth	++	Medium
FOC-5	White with pale violet	Fluffy, smooth	++	Medium
FOC-6	White with pale violet	Fluffy	++	Medium
FOC-7	Dark brown with white	Fluffy, smooth	+++	Medium
FOC-8	White with pale violet	Fluffy, smooth	++	Slow
FOC-9	Creamy white	Fluffy	++	Slow
FOC-10	Creamy with dark violet	Fluffy, smooth	+	Slow
FOC-11	Pale pink	Smooth	+	Slow
FOC-12	Creamy white with pale pink	Fluffy, smooth	++	Medium
FOC-13	Creamy white with dark violet	Smooth	+++	Slow
FOC-14	Dark brown with white	Fluffy	+++	Slow
FOC-15	Violet	Smooth	+	Slow
FOC 16	Creamy white with pale pink	Fluffy	+++	Fast
FOC-17	Creamy white with dark violet	Fluffy	+	Slow
FOC-18	Creamy white with dark pink	Fluffy	++++	Fast
FOC-19	Creamy white with pale pink	Fluffy	++++	Fast
FOC-20	White with pale violet	Fluffy, smooth	+	Slow
FOC-21	White with pale violet	Fluffy	++++	Fast
FOC-22	Dark brown with white	Fluffy	+++	Fast
FOC-23	White with violet	Fluffy	++++	Fast
FOC-24	White with dark pink	Fluffy	++++	Fast
FOC-25	Milky white with light yellow	Fluffy	+++	Fast
FOC-26	Milky white with light brown	Fluffy	+++	Medium
FOC-27	Milky white with dark pink	Fluffy, smooth	++++	Fast
FOC-28	Dark brown with white	Fluffy	+++	Fast
FOC-29	Milky white with pale violet	Fluffy, smooth	+++	Fast
FOC-30	Creamy white with pale violet	Fluffy	+++	Medium

(+) Poor sporulation, 1-75 spores/microscopic field (40X); (++) Medium sporulation, 76-150 spores/microscopic field (40X); (+++) Good sporulation, 151-226 spores/microscopic field (40X) and (++++) High sporulation, more than 227 spores/microscopic field (40X).

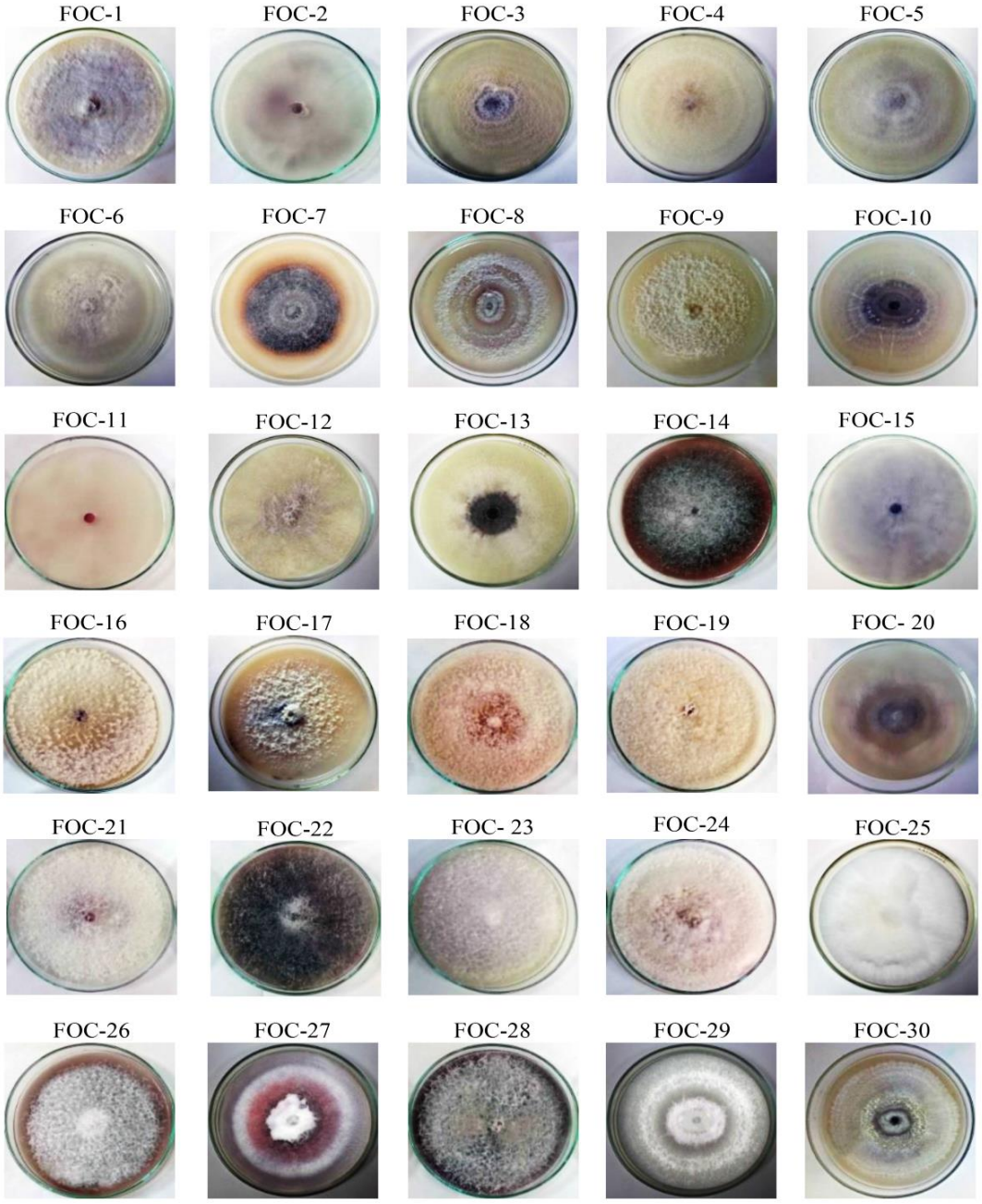

Figure (2): Mycelial growth of *F. oxysporum* f. sp. *cucumerinum* isolates.

4- Disease symptoms, pathogenicity and virulence of FOC isolates:

All the tested isolates caused significant wilt symptoms in cucumber plants at 15 days after inoculation under greenhouse conditions. The symptoms started with yellowing of lower plant leaves, necrosis and discoloration of both root and stem xylem vessels and finally death of plants. Data presented in Table 3 show that all tested isolates of FOC were able to infect the susceptible cucumber cultivar Beit Alpha. Degrees of disease severity varied from weak to severe with significant differences. Isolates FOC-25, FOC-18 and FOC-24 recorded the highest percentages of disease severity, being 100.0, 95.3 and 90.0%, respectively. While the lowest percentages of disease severity were reported for isolates FOC-11, FOC-15 and FOC-17 that gave 7.5, 10.8 and 13.7%, respectively. Isolates FOC-13, FOC-22, FOC-26 recorded the moderate percentage of disease severity 40.4, 42.3 and 45.7%, respectively. The very week isolate FOC-27 recorded 3.0% disease severity. On the other hand, isolates were divided into four groups of

aggressiveness degree based on the disease quantum as follows, slightly aggressive isolates FOC-27, 11, 15, 17, 10, 20, 2, 6, 4, 12, 5, 9, 8; moderately aggressive isolates FOC-30, 7, 14, 3, 29, 28, 13, 22, 26; highly aggressive isolates, FOC-16, 23, 1 and extremely aggressive isolates FOC-21, 19, 24, 18, 25.Moreover, isolate FOC-25 recorded the maximum percentage of disease severity whereas FOC-27 isolate recorded the lowest percentage of disease severity (Table 3). The effect of FOC isolates on cucumber growth was analyzed. Plant height, root and shoot fresh & dry weights were measured (Table 4). All isolates showed various reduction values in all growth measurements. FOC-25 followed by FOC-18 and FOC-24 recorded the highest decrease in all measurements, while FOC-11 followed by FOC-15 and FOC-17 recorded the lowest decrease in all measurements comparing with control. The isolate FOC-27 recorded very slightly effect. There is a reverse relationship between cucumber productivity and aggressiveness of FOC isolates, the highest negative effect was recorded by isolates FOC-25, 24 and 18 sequentially, while the lowest negative effect was given by isolate FOC-27.

Table (3): Average of disease severity assessment and aggressiveness degree of FOC isolates surveyed from 2 locations (Qallin & Kafr El-Sheikh) on cucumber cv Beit Alpha after 15- and 30-days post-inoculation under greenhouse conditions.

Isolate code	Isolate location	Disease severity %		Aggressiveness degree
		45 days	60 days	
FOC-1	Kafr El-Sheikh	10.5	60.7	Highly aggressive
FOC-2	Kafr El-Sheikh	6.00	15.5	Slightly aggressive
FOC-3	Kafr El-Sheikh	8.70	35.5	Moderately aggressive
FOC-4	Kafr El-Sheikh	6.80	17.5	Slightly aggressive
FOC-5	Kafr El-Sheikh	7.50	20.0	Slightly aggressive
FOC-6	Kafr El-Sheikh	6.30	15.6	Slightly aggressive
FOC-7	Kafr El-Sheikh	8.50	30.5	Moderately aggressive
FOC-8	Kafr El-Sheikh	7.80	22.5	Slightly aggressive
FOC-9	Kafr El-Sheikh	7.60	20.7	Slightly aggressive
FOC-10	Kafr El-Sheikh	6.40	14.5	Slightly aggressive
FOC-11	Qallin	3.20	7.50	Slightly aggressive
FOC-12	Qallin	7.10	19.5	Slightly aggressive
FOC-13	Kafr El-Sheikh	8.90	40.4	Moderately aggressive
FOC-14	Kafr El-Sheikh	8.50	30.3	Moderately aggressive
FOC-15	Qallin	5.30	10.8	Slightly aggressive
FOC-16	Qallin	9.80	55.5	Highly aggressive
FOC-17	Qallin	6.20	13.7	Slightly aggressive
FOC-18	Kafr El-Sheikh	32.4	95.3	Extremely aggressive
FOC-19	Kafr El-Sheikh	30.4	88.4	Extremely aggressive
FOC-20	Qallin	6.40	15.3	Slightly aggressive
FOC-21	Kafr El-Sheikh	26.9	82.3	Extremely aggressive
FOC-22	Qallin	9.10	42.3	Moderately aggressive
FOC-23	Kafr El-Sheikh	10.3	60.0	Highly aggressive
FOC-24	Qallin	30.9	90.0	Extremely aggressive
FOC-25	Qallin	55.6	100	Extremely aggressive
FOC-26	Qallin	9.40	45.7	Moderately aggressive
FOC-27	Qallin	0.00	3.00	Slightly aggressive
FOC-28	Qallin	8.80	37.3	Moderately aggressive
FOC-29	Qallin	8.80	39.7	Moderately aggressive
FOC-30	Qallin	8.20	27.3	Moderately aggressive
Control	---	0.00	0.00	---
LSD at 0.05	---	1.287	3.752	---

Table (4): Parameters of cucumber plants: plant height, fresh & dry weights, and fruit production after 30 days post-inoculation under greenhouse conditions.

Isolate code	Plant height (cm)	Fresh weight (gm)		Dry weight (gm)		Plant product (kg)
		Root	Shoot	Root	Shoot	
FOC-1	69.3	3.1	13.2	0.28	1.36	0.35
FOC-2	67.3	5.2	15.0	0.38	2.26	0.45
FOC-3	65.9	4.4	14.5	0.35	1.90	0.41
FOC-4	66.9	5.1	15.0	0.38	2.22	0.45
FOC-5	69.2	4.9	14.7	0.37	2.15	0.44
FOC-6	67.2	5.1	15.0	0.38	2.24	0.45
FOC-7	66.4	4.5	14.7	0.36	2.00	0.42
FOC-8	67.2	4.8	14.2	0.37	2.13	0.43
FOC-9	69.0	4.9	14.7	0.37	2.15	0.44
FOC-10	68.4	5.2	15.3	0.38	2.27	0.46
FOC-11	71.4	5.6	15.7	0.40	2.32	0.48
FOC-12	66.2	4.9	14.9	0.37	2.16	0.44
FOC-13	65.0	4.0	14.6	0.34	1.70	0.39
FOC-14	66.8	4.5	14.8	0.36	2.05	0.42
FOC-15	68.8	5.5	15.5	0.39	2.30	0.48
FOC-16	60.3	3.4	13.8	0.30	1.50	0.37
FOC-17	67.9	5.4	15.2	0.39	2.29	0.47
FOC-18	38.3	2.3	12.0	0.23	0.85	0.25
FOC-19	45.3	2.6	12.3	0.24	1.23	0.28
FOC-20	67.9	5.2	15.0	0.38	2.26	0.46
FOC-21	49.1	3.1	12.7	0.27	1.38	0.32
FOC-22	64.3	3.8	14.4	0.33	1.63	0.39
FOC-23	59.7	3.1	13.2	0.28	1.38	0.36
FOC-24	44.2	2.4	12.1	0.24	1.14	0.25
FOC-25	31.2	2.1	11.2	0.2	0.80	0.23
FOC-26	63.7	3.7	14.2	0.33	1.60	0.38
FOC-27	75.2	6.3	16.4	0.63	3.10	0.72
FOC-28	65.5	4.3	15.0	0.35	1.80	0.40
FOC-29	65.1	4.2	14.7	0.34	1.80	0.39
FOC-30	67.0	4.6	15.1	0.36	2.10	0.43
Control	75.8	6.5	16.7	0.67	3.40	0.75
LSD at 0.05	3.163	0.466	0.997	0.021	0.158	0.038

5- Genetic similarities and phylogenetic analysis based on RAPD:

Genomic DNA isolated from 30 isolates of FOC was subjected to RAPD PCR analysis with 15 random decamer primers. Six out of the fifteen primers tested produced polymorphic bands; 3 of them were used for a comparative analysis of the 30 isolates of FOC. These primers were FOCP-1, FOCP-5 and FOCP-7 (Figure 3). Two primers generated reproducible band profiles in all isolates FOCP-8 and FOCP-10. However, 7 primers did not generate bands in all isolates; they gave amplification in some isolates and did not give amplification in the others. Amplified fragments ranged from 450 bp to 1.8 kb. The DNA fragments generated by primer 1, 5 and 7 proved a unique profile for isolates FOC-25 and FOC-27. The number of DNA fragments, amplified and scored per isolate for individual primer, ranged from 2 to 5. The RAPD analysis revealed a high degree of genetic variation among the isolates. The cluster analysis based on DNA fragments generated by Primers 1, 5, 7 gave two clusters; the first cluster included the highly and extremely aggressive isolates. The second cluster was divided into 2 sub-clusters; the first sub-cluster contained the moderately aggressive isolates while the second sub-cluster contained the slightly aggressive isolates (Figure 4).

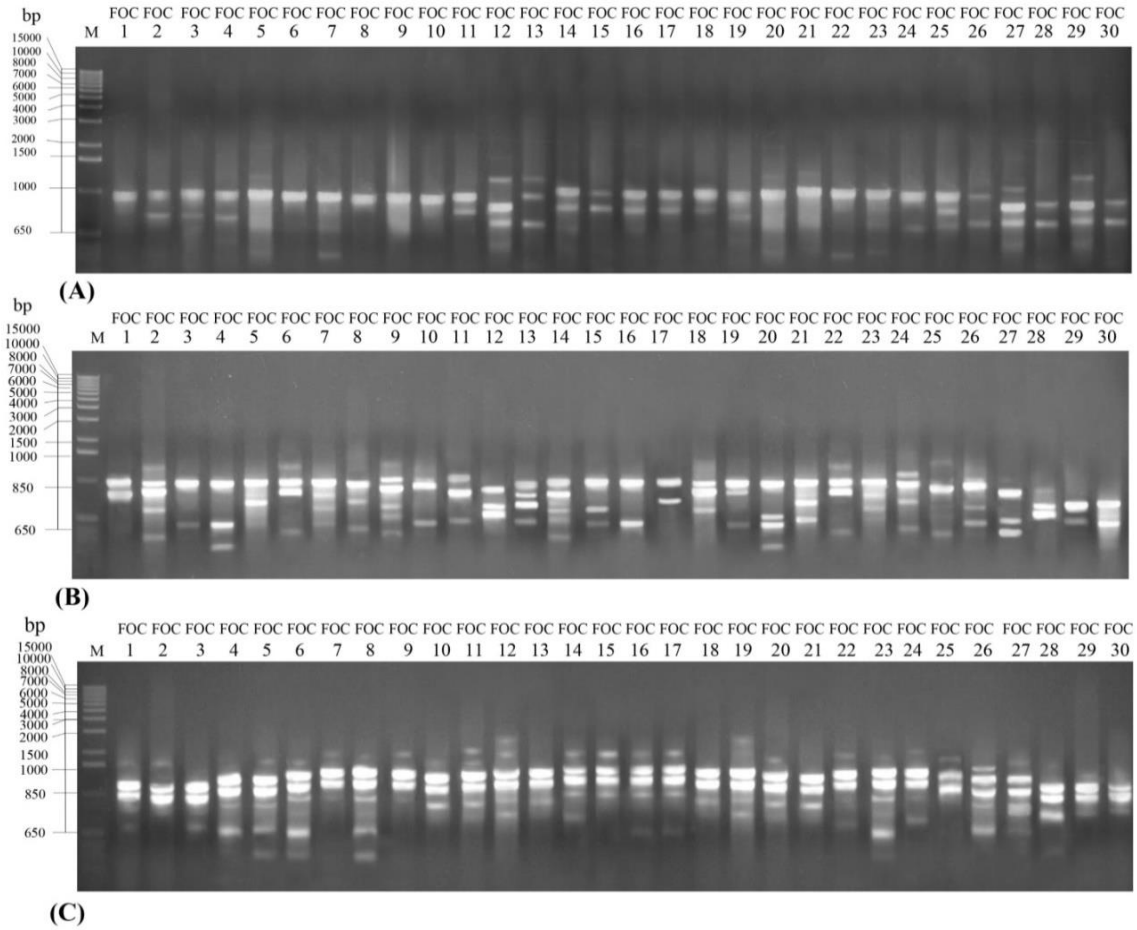

Figure (3): RAPD marker profiles of *Fusarium oxysporum* f. sp. *cucumerinum* isolates generated by primers (A) FOCP-1, (B) FOCP-5 and (C) FOCP-7 in 2% agarose gel.

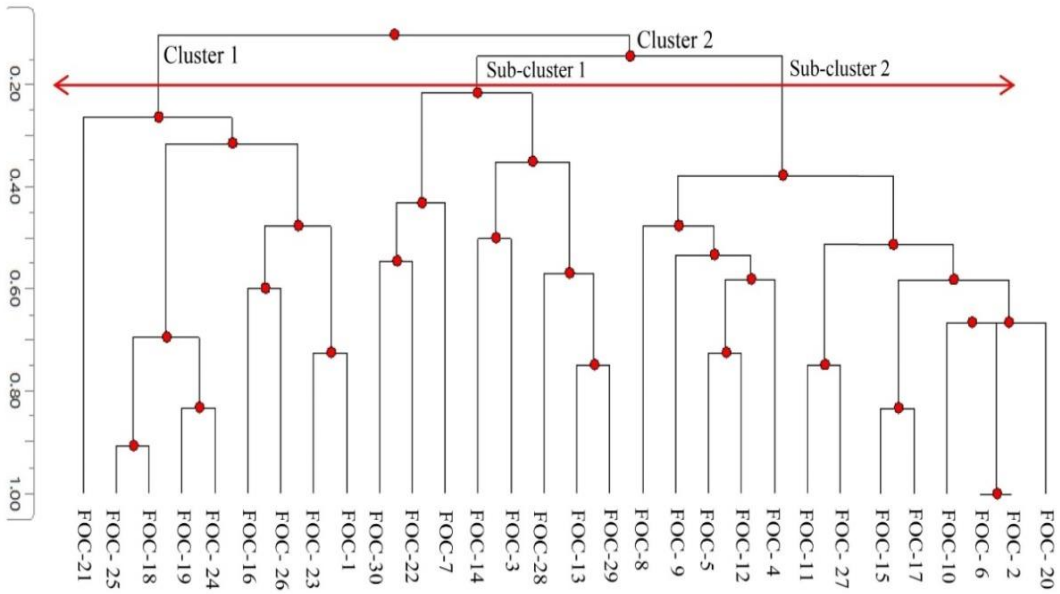

Figure (4): Dendrogram showing the cluster analysis of *Fusarium oxysporum* f. sp. *cucumerinum* isolates using primers 1, 5 and 7.

6- Protein Profile of FOC Isolates

The mycelial proteins of the studied isolates of FOC were electrophoreticaly analyzed on SDS-PAGE (Figure 5a). The electrophoretic patterns showed a great number of polymorphic bands with molecular weights ranged from 10 to 175 KDa. The high and low pathogenic isolates showed complete variation in protein profile. There was a definite relation between the number and the intensity of protein bands and pathogenicity. The cluster analysis separated the isolates into 3 main clusters, the first and second clusters include mixture of slightly and moderately aggressive isolates, while the third cluster divided into 2 sub-clusters, first one included moderately aggressive isolates and second one included highly and extremely aggressive isolates (Figure 5b).

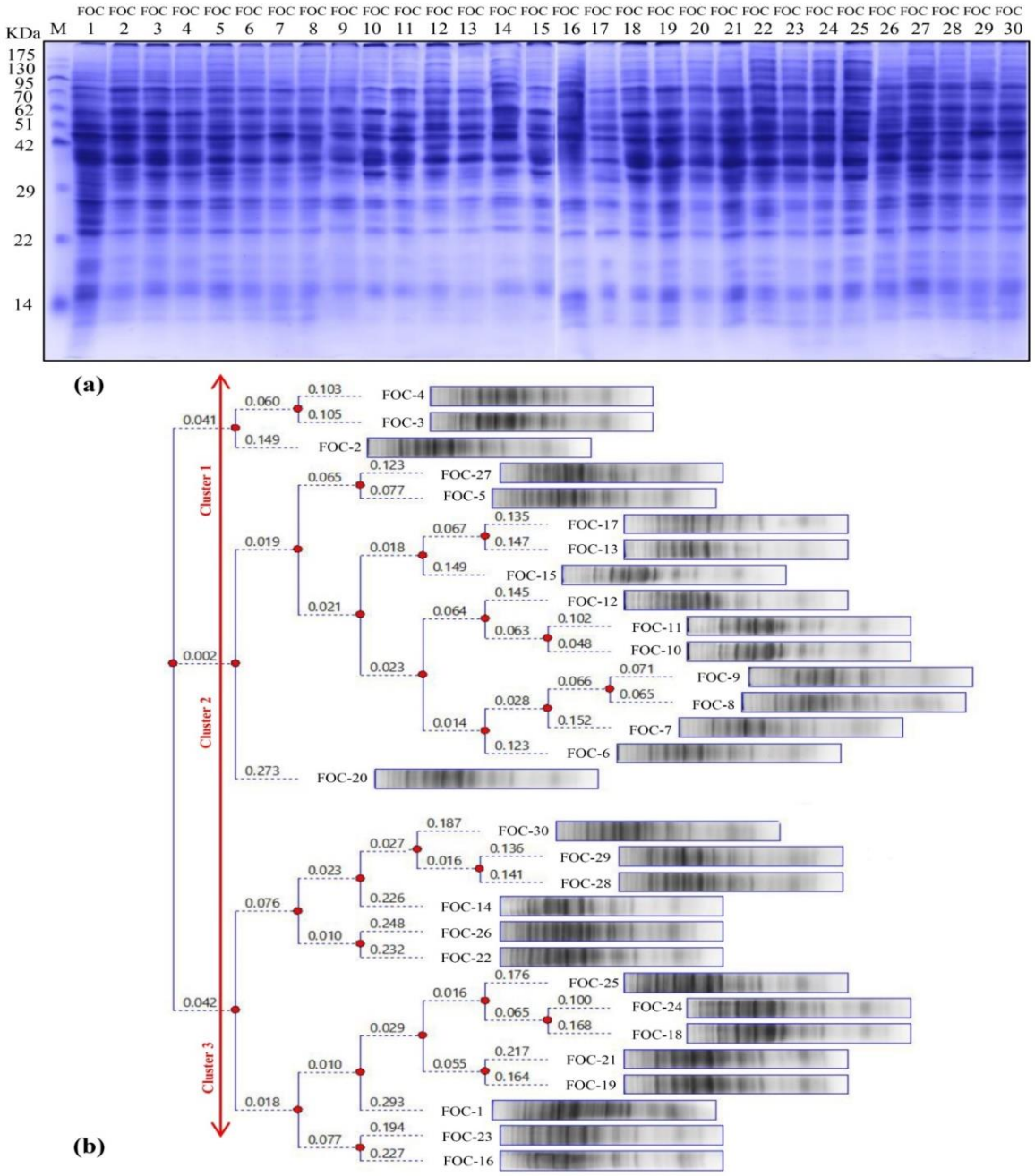

Figure (5): Protein profile of *F. oxysporum* f. sp. *cucumerinum* isolates. a) SDS-PAGE analysis of isolates, b) dendrogram derived from SDS-PAGE analysis of isolates.

DISCUSSION

Fusarium oxysporum f. sp. *cucumerinum* isolates were surveyed from hotspot adjacent zones in Kafr El-Sheikh, governorate, Egypt, determined based on the disease history, epidemiology and aggressiveness in these zones. It is assumed that the surveyed isolates from the same location have similar morphological characteristics, but our results showed great morphological variation in the isolates despite they were collected from closely related fields. This morphological variation is due to fungal adaptation and selective pressure caused by excessive and indiscriminate use of fungicides, the cultivation of the same crop in the same area for consecutive years and the changes in climatic conditions. All the previous factors caused mutations in the Fusarium isolates, resulting in new more virulent pathogenic strains with high variation in their morphological characteristics.

Our FOC isolates were highly variable in their mycelial growth pattern, colony size, growth rate, sporulation degree and variation in pigments. These results are compatible with the results recorded by Dubey *et al*. (2010); Arvayo-Ortiz *et al*. (2011) and Manikandan *et al*. (2018). Our disagreement with them is that the differences among isolates are not due to collecting them from places far apart between them as they mentioned in their studies but the differences are due to the reasons and the factors previously mentioned. Our study showed a positive relation between the amount of spore production and virulence of isolates. The isolates produced abundant sporulation were highly virulent, while poor to moderately sporulated isolates produced low percent of pathogenicity or were nonpathogenic (Golakiya *et al*., 2018). We observed that FOC-27 produced high spore production and showed a minimum degree of pathogenicity to maintain its survival life. This isolate may has antagonistic activity that can be used to reduce the vascular wilt disease severity, this is compatible with the study done by Silva and Bettiol (2005), who studied the efficiency of the non-pathogenic *F. oxysporum* isolates in controlling Fusarium wilt in tomato.

Pathogenicity results showed that all 30 FOC isolates have a pathogenic ability in cucumber plants and cause similar wilt symptoms. *Fusarium* isolates cause vascular discoloration and blockage in xylem vessels, through the production of enzymes which decompose cellulosic and pectin components of plant cell walls that effect on cell permeability (Talboys, 1957). Toxins also play important role in inducing symptoms and changing physiological processes in wilted plants such as activation of polyphenol oxidases, increasing growth regulators activity and respiration rate and alteration in phenolic metabolism (Davis, 1954; MacHardy and Beckman, 1981). Furthermore, infected plants form callose barriers and tylose-like structures to prevent FOC invasion inside the xylem vessels with the presence of fungal mycelium mass (Street and Cooper, 1984; Rodríguez-Gálvez and Mendgen, 1995; Mepsted *et al*., 1995; Trillas, 2000). Consequently, the flow of water inside the plant significantly decreased and the water absorption reduced.

All previous distinctive responses explain the lack of water inside the plant and consider it the important causes of Fusarium wilting. Due to the variation and overlapping in morphological characteristics between isolates, it is difficult to rely on them to explain the differences in their pathogenicity and genetic variation. Scientists have tended to explain differences of isolates based on protein and DNA profiles as they reflect the physiological state and morphological structure of the isolates and gives an explanation of the complex interactions that control the development of pathogens (Guarro *et al*., 1999). The isolates grouped under the same aggressiveness degree showed the same electrophoretic protein patterns which reflect the morphological characters and physiological state of the isolate. There is a variation in the position of bands (qualitative) and the number of bands (quantitative) between high and low pathogenic isolates due to the expression of the avirulence (*Avr*) gene.

We found that all isolates are genetically different due to the direct and indirect human interference in changing the natural conditions around plant and pathogen such as frequent use of fungicides; bio-agents and inducers under the name of improving and developing the environment leading to hereditary changes and emergence of genetic mutations which were mostly favorable for the pathogen. These changes allowed the pathogen to produce new strains that are highly pathogenic and more aggressive; this explains the existence of Fusarium epidemic that

caused heavy losses in the productivity in the area under study.

CONCLUSION

The frequent *Fusarium* isolates obtained from cucumber plants were identified as *F. oxysporum* f. sp. *cucumerinum*. The isolates showed high morphological variations and genetic diversity; in spite of collecting them from the same geographical area. The isolate FOC-25 was more virulent while, FOC-27 was slightly aggressive more than the other 28 isolates tested. The other prospect we intend to do in the future is a comprehensive study to assess the diversity at the proteomics level between high-pathogenic and weak-pathogenic isolates.

List of primers

Name	Primer sequence
FOC-1	5′-AAACGAGCCC-′3
FOC-2	5′-CCGAATTCCC-′3
FOC-3	5′-GATGACCGCC-′3
FOC-4	5′-GGACCCTTAC-′3
FOC-5	5′-TGCCGAGCTG-′3
FOC-6	5′-GTGATCGCAG-′3
FOC-7	5′-CATCCCCCTG-′3
FOC-8	5′-AGGTGACCGT-′3
FOC-9	5′-GGACTGGAGT-′3
FOC-10	5′-GGAGGGTGTT-′3
FOC-11	5′-GGTGACGCAG-′3
FOC-12	5′-GTCCACACGG-′3
FOC-13	5′-GTTTCGCTCC-′3
FOC-14	5′-TCCGCTCTGG-′3
FOC-15	5′-TGATCCCTGG-′3

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AUTHOR CONTRIBUTIONS

Elagamey, E. conceived and designed the experiments; Kamel, S.M. and Essa, T.A. collected the isolates; Elagamey, E., Abdellatef, M.A.E., Kamel, S.M. and Essa, T.A. performed the experiments. Elagamey, E., Abdellatef, M.A.E. and Kamel, S.M. carried out the data analysis. Elagamey, E. and Abdellatef, M.A.E. discussed the study and wrote the article. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST:

The authors declare no conflict of interest exists.

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