Differentiation between Tomato Fusarium Wilt Isolates by ISSR Markers and Virulence Analysis

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DOI: 10.21608/JALEXU.2022.168893.1090

Article Information

Received:October 15th 2022

Revised: November 13th 2022

Accepted: November 22nd 2022

Published: December 31st 2022

ABSTRACT: *Fusarium oxysporum.* f. sp. *lycopersici* (FOT) causes tomato fusarium wilt, one of the critical fungal diseases causing severe losses in tomato production in Egypt. Eight isolates of *F. oxysporum*. f. sp. *lycopersici* were isolated from infected tomato plants collected from six Egyptian governorates. FOT4, FOT2, and FOT5 isolates were highly virulent, while FOT6 and FOT8 were moderately virulent, and FOT7, FOT3, and FOT1 were weakly virulent. All tested ISSRs primers were polymorphic and produced 81 DNA loci of various molecular sizes. DNA fragment patterns analysis showed 37 monomorphic and 44 polymorphic bands, with 54.32% polymorphism between the tested isolates. Among the tested isolates, primer UMC-840 had the highest level of polymorphism (85.71%), while primer HB-13 had the lowest level (33.33%). Dendrogram and ISSR band patterns were analyzed using the UPGMA method to detect the relationship between the eight isolates. The highest genetic homology (82.9%) was observed between FOT4 and FOT3 isolates, while the lowest genetic homology (63.5%) was observed between FOT1 and FOT2 isolates. The eight isolates were classified into three main clusters (A, B, and C). Cluster A includes two sub-clusters; sub-cluster A1 is divided into two groups, group1 consists of two subgroups, subgroup1 include FOT 6 isolate, and subgroup 2 includes FOT4, FOT3, and FOT5 isolates (FOT4 and FOT5 were high virulent isolates), group2 has FOT8 isolate. Sub-cluster A2 includes FOT6 isolate. However, cluster B includes the FOT1 isolate, and cluster C includes the FOT2 isolate.

Keywords: *Fusarium, isolates, polymorphism, dendrogram, and ISSR*

INTRODUCTION

Fusarium species cause various diseases in many host plants (Summerell *et al.*, 2003; Booth, 1971). These soilborne fungi are economically crucial because many of their members cause root rot and vascular wilt diseases in cultivated crops worldwide (Woo *et al.*, 1998). Because of its global distribution in soil, *F. oxysporum* has been dubbed the global mycoflora (Parkinson, 1981). Fusarium species are commonly identified using their macro and microscopic characteristics. However, these characteristics are generally reported to be unstable (Szecsi and Dobrovolsky, 1983; Nelson *et al.*, 1985). The morphological, biochemical, and allozyme characteristics are the most commonly used methods for pathogens identification, which require professional experience and are still subject to error (Kheterpal, 2006). Currently, pathogens identification is based on nucleotide sequence and genetic markers analysis. DNA markers used in genetic variation analysis are characterized by high genetic divergence and the ability to detect multilocus data in the genome (Anne, 2006). Molecular markers such as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Cleaved Amplified Polymorphic Sequences (CAPs), and Inter Simple Sequence Repeats (ISSR) are widely used in constructing fungi genetic maps. ISSR markers amplify microsatellite DNA sequences, which are highly varied and widespread in the genome. ISSR is an efficient multilocus technique that enables scoring multiple polymorphic loci and does not need any previous sequence information; however, it is limited by repeatability issues. Furthermore, the ISSR technique has high stability, accuracy, and efficiency compared to morphological assessment, so it has been widely used in genetic diversity studies (Williams *et al.*, 1990; Guo *et al.*, 2001). Troncoso-Rojas *et al.* (2013) use ISSR and RAPD makers to evaluate the genetic diversity and genotyping of *Alternaria solani* isolates. DNA markers such as RFLP, AFLP, RAPD, ISSRs, simple sequence repeats (SSR), and expressed sequence tags (EST) were used in the analysis of the genetic variation in various *F. oxysporum* formae speciales (Bogale *et*

al., 2005; Dubey and Singh 2008; Dubey *et al.*, 2010; Sharma *et al.*, 2014; Yuan *et al.*, 2013). Singh and Kapoor (2018) used ISSR markers to develop sequence-characterized amplified region (SCAR) markers to identify and quantify *Fusarium oxysporum* f. sp. *carthami*. Molecular tools are the most accurate for identifying plant pathogens. Their use has increased in recent years due to the availability of specific equipment and chemicals and their ability to identify the types and aggressiveness of isolates (Adss *et al.*, 2017). This study aimed to isolate, purify and identify *F. oxysporum.* f. sp. *lycopersici* isolates from six governorates of Egypt and differentiate these isolated using ISSR- PCR and virulence test.

MATERIALS AND METHODS

1. The causal fungal sample collections

Eight fungal isolates were isolated from infected tomato plants with typical wilt symptoms collected from six governorates in Egypt; El-Menofia (Talla and Shebeen Elkom), El-Menia, Assiut, Kafr El-Sheikh, Alexandria, El-Beheira (Abou-Homus and Kom Hamada).

2. Isolation and identification of the causal fungi

The collected tomato root rot samples were thoroughly washed in tap water and air-dried. Small pieces were taken from the internal tissues adjacent to the affected root parts, surface sterilized in 3 % sodium hypochlorite solution for 3 mins., rinsed in sterile distilled water, and dried between sterilized filter papers. After that, samples were transferred to PDA plates containing streptomycin sulphate (0.2g/L) and incubated at 28ºC for five days (Shaukat *et al.*, 2005). The hyphal tip technique was used to purify the isolated fungi, as described by Brown (1924). Identifications of the causal fungi were made at the Department of Plant Pathology, Faculty of Agriculture, Damanhour university, according to Gilman (1957), Singh (1982), and Summerell *et al.* (2003). The frequency of the fungal isolates recovered from the surveyed governorates was calculated

3. Pathogenicity test

The eight *F. oxysporum* f. sp. *lycopersici* isolates were tested for pathogenicity on the Carmen tomato cultivar. Tomato seeds were sterilized for five min. in 1% sodium hypochlorite, rinsed with sterile water, and sown in 20cm diameter plastic pots filled with 3Kg sterilized peat moss, clay, and sand (1:1:1). For Fungi inocula preparations, pathogens were cultured on Potato Dextrose Broth (PDB) medium in 250ml conical flasks and incubated at 25 ± 2°C for 10 days. The mycelial cultures were then filtered, and the spore suspensions were adjusted to 10^6 conidia/ml using a haemacytometer slide. Plants were inoculated when they reached 15-20cm tall by adding 50 ml of the prepared conidial suspension to 1kg of soil (Elad and Baker, 1985).

4. Disease assessments

The severity of wilting was rated 15-day post-inoculation (dpi) using a pathogenicity scale of 6 grades, according to Liu *et al.* (1995) (Table 1).

Disease severity (%) = {Σ (Rating no x no. plants in rating category) / (Total no. plants x highest rating value)} × 100

Table 1. Assessment scale of disease severity of *F. oxysporum* of tomato according to Liu *et al.* (1995).

Category	Grade	% of Infected Leaf Area
I	0	no symptoms
II	1	Plants with < 25% of diseased leaves
III	2	Plants with 25-50% of diseased leaves
IV	3	Plants with 50-75% of diseased leaves
V	4	Plants with 76-100% of diseased leaves
VI	5	Plants completely died.

5. Differentiation between *F. oxysporum* isolates using ISSR analysis

5.1. Fungal DNA extraction

Fungal genomic DNA was isolated by Wizard Genomic DNA purification kit (Promega, Germany) according to the manufacturer's procedures.

5.2. ISSR reactions

PCR amplification was performed using nine ISSR primers (Table 2) to differentiate and fingerprint the *F. oxysporum* isolates. PCR reaction (25µl) contained 10x PCR buffer (2.5µl), 4mM dNTPs (2.5µl), 50mM $MgCl_2$ (2.5µl), 50pmol primer (7µl), 50ng of fungal genomic DNA (1µl), and 5 units/µl Taq DNA polymerase (0.2µl) (Promega, Germany). The PCR program was: initial denaturation at 95ºC for 5 min, then 40 cycles of 95ºC for 1 min, annealing at a temperature depending on primer for 1 min (Table 2), extension at 72ºC for 1 min, and a final extension at 72ºC for 10 min (Istock *et al.*, 2001). The amplified DNA products were electrophoresed on 1.5% (w/v) agarose gel in 0.5 x TBE buffer, stained with ethidium bromide (0.5 µg/cm3, w/v), and photographed using a gel documentation system.

Table 2. ISSR primers sequences and annealing temperature used in this study.

Primer	Nucleotide sequence (5′ to 3′)	Annealing Temperature ºC
HB-09	GTGTGTGTGTGTGG	55
HB-11	GTGTGTGTGTGTCC	52
HB-12	CACCACCACGC	51
HB-13	GAGGAGGAGGC	50
HB-14	GTGTGTGTGTGTGC	55
UBC814	ACACACACACACACACC	55
UBC835	AGAGAGAGAGAGAGAGAC	60
UBC840	GAGAGAGAGAGAGAGATT	55
UBC868	GAAGAAGAAGAAGAAGAA	51

5.3. Construction of dendrogram based on ISSR-PCR DNA banding patterns.

ISSR-PCR banding patterns for each primer were recorded depending on the presence and absence of the amplified bands. A band is scored as (1) or (0), depending on whether it is present or absent. Multi-Variate Statistical Package (MVSP) Version 3.1 was used to compare the *F. oxysporum* isolates and the unweighted pair group method with arithmetic averages (UPGMA) used to compute the similarity coefficients and to conduct cluster analysis.

6. Statistical analysis

Disease severity data were analyzed with SAS ver. 9.4 (SAS Institute Inc., Cary, NC, USA), and the least significant difference (LSD) test was used to differentiate means at a $p \leq 0.05$.

RESULTS AND DISCUSSIONS

F. oxysporum. f. sp. *lycopersici* is the disease agent of tomato fusarium wilt and one of the most critical tomato pathogens, and it causes significant damage and economically severe crop losses, particularly at high temperatures and humidity in the indoor environment (Jones *et al.,* 1982; Smith *et al.,* 1988; Agrios, 2005).

1. Isolation and Identification of *F. oxysporum* isolates

Eight isolates of *F. oxysporum* (FOT) were obtained and identified from tomato-infected plants (Table 3) collected from El-Menofia (Talla (FOT1) and Shebeen Elkom (FOT2)), El-Menia (FOT3), Assiut (FOT4), Kafr El-Sheikh (FOT5), Alexandria (FOT6), El-Beheira governorate (Abou-Homus (FOT7) and Kom Hamada (FOT8)).

2. Pathogenicity test

The eight *F. oxysporum* isolates were tested for pathogenicity on the highly susceptible tomato cultivar Carmen. Data presented in Table (3) and Fig (1) showed that all eight isolates were virulent. Isolates FOT4 from Assiut, FOT2 from El-Menofia (Shebeen Elkom), and FOT5 from Kafr El-Sheikh were highly virulent. In comparison, isolates FOT6 from Alexandria and FOT8 from El-Beheira (Kom Hamada) were moderately virulent. While isolates FOT7 from Abou Homus, FOT3 from El-Menia and FOT1 from El-Menofia (Talla) were weakly virulent. These results agreed with those of Abou-Zeid *et al.* (2016). They obtained six *F. oxysporum* isolates from diseased pepper collected from Gharbya and Dakahlya governorates in Egypt. The F3 isolate from Dakahlya was more aggressive (96.67% wilt severity) than Tanta F4 isolate (71.67% severity).

Table 3. The pathogenicity test of eight *F. oxysporum.* f. sp. *lycopersici* isolates on Carmen tomato cultivar

Treatment	Disease severizty* (%)
FOT4	75[a]
FOT2	70[a]
FOT5	68[a]
FOT6	50[b]
FOT8	46[b]
FOT7	30[c]
FOT3	25[c]
FOT1	13[d]

*Data are an average of three replicates. $LSD_{0.05}$ for fungal isolates = 8.24

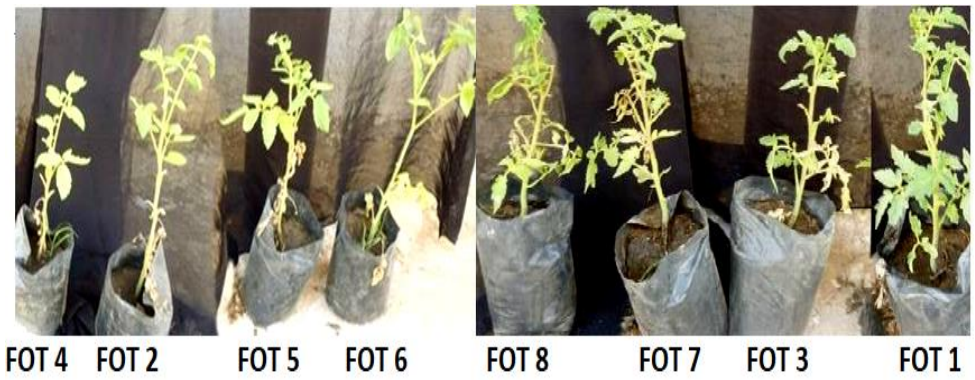

Fig. 1. Reaction of Carmen tomato cultivar to the infection with eight *F. oxysporum.* f. sp. *lycopersici* isolates

3. Fingerprinting of *F. oxysporum* isolates using ISSR markers

3.1. ISSR banding pattern analysis

Genetic diversity analysis of the eight *F. oxysporum* isolates using ISSR markers revealed a high variability level among these isolates. The nine tested ISSRs primers (UBC814, UBC840, UBC808, UBC811, UBC868, HB-11, HB-12, and HB-13) were polymorphic, and successfully generated 81 DNA loci with a molecular size ranging between 130 -1500bp (Fig. 2). DNA banding pattern analysis showed 37 monomorphic and 44 polymorphic bands, giving 54.32% polymorphism among the tested isolates. Primer UMC-840 had the highest level of polymorphism (85.71%) among the tested isolates, while primer HB-13 had the lowest level (33.33%).

Primer UBC814 yielded 57 bands found in nine loci with molecular sizes ranging between 150 -1400bp. Five of the nine observed loci were polymorphic, with 55.55% Polymorphism between isolates. This primer provided the lowest numbers of bands (6 bands) with isolates FOT3, FOT5, and FOT8, while the highest numbers (9 bands) were observed with FOT4 isolates. Primer UBC840 generated 37 fragments expressed in seven loci ranging from 160 to 1400bp. Six of these loci were polymorphic, and the highest level of polymorphism among the isolates (85.71%) was observed with this primer.

Primer UBC868 produced 54 fragments presented in nine loci with molecular weights ranging between 150- 1000bp. Five of the nine detected loci were polymorphic, with 55.55% polymorphism. This primer yielded the lowest numbers of bands (6 bands) with FOT7, FOT5, FOT6, and FOT 4 isolates, while it produced the highest (8 bands) with FOT1 and FOT2 isolates. Primer UBC835 generated 48 fragments phrased in 9 loci with different molecular sizes (130-1500bp). The polymorphism level of this primer was 77.77%, with seven of the nine observed loci being polymorphic. The lowest numbers of derived bands (5 bands) were noted with isolates FOT 6, while the highest numbers (9 bands) were noted with the FOT7 isolate.

Primer UB-11 produced 47 bands expressed in nine loci with molecular weights ranging from 200 to 1200bp. This primer had a polymorphism level of 55.55% with five polymorphic loci. The highest number of obtained bands (8 bands) were detected with the FOT7 isolate, while the lowest number (5 bands) were detected with FOT1 and FOT6 isolates. Primer UB-13 generated 65 fragments in nine loci ranging from 130 to 970bp. Three of the nine observed loci were considered polymorphic, and the lowest level of polymorphism among the tested isolates (33.33%) was observed with this primer. Isolates FOT1 and FOT4 had the lowest numbers of generated bands (7 bands), while isolates FOT2, FOT3, and FOT7 had the highest (8 bands).

Primer UB-09 amplified 48 fragments in 9 loci with molecular weights ranging between 150 and 900bp. Five of the nine detected loci were polymorphic, with 55.55% polymorphism. This primer generated five bands with FOT6 isolate and eight bands with FOT7 isolate. Primer UB-12 yielded 52 bands in eight loci with molecular sizes ranging from 180 to 970bp. Four of these observed loci were considered polymorphic. The level of polymorphism among the tested isolates was 50%. This primer produced five bands with isolates FOT7, while it produced eight bands with FOT4 isolate. Primer UB-14 generated 35 fragments in six loci with molecular sizes ranging between 190 and 700bp. Three of the six detected loci were polymorphic, with a polymorphism level of 50%. Three bands were recorded with the FOT1 isolate, while six bands were recorded with the FOT4 isolate.

These findings are consistent with previous research that found ISSR markers to be helpful in assessing the genetic variability of *F. oxysporum* (Bayraktar *et al.,* 2008; Dubey and Singh, 2008; Baysal *et al.,* 2010; Thangavelu *et al.,*

2012). ISSR markers produce higher polymorphism because they amplify the conserved regions between the microsatellite repeat sequences, which are widely distributed in the genome (Zietkiewicz *et al.*, 1994). Differences in ISSR polymorphism were also found in Fusarium strains isolated from either cabbage or tomato (Troncoso-Rojas *et al.*, 2013). Singh *et al*. (2019) reported high genetic diversity in 90 isolates of *F. oxysporum f. sp. carthami* isolated from Safflower using 17 polymorphic RAPD and ISSR primers.

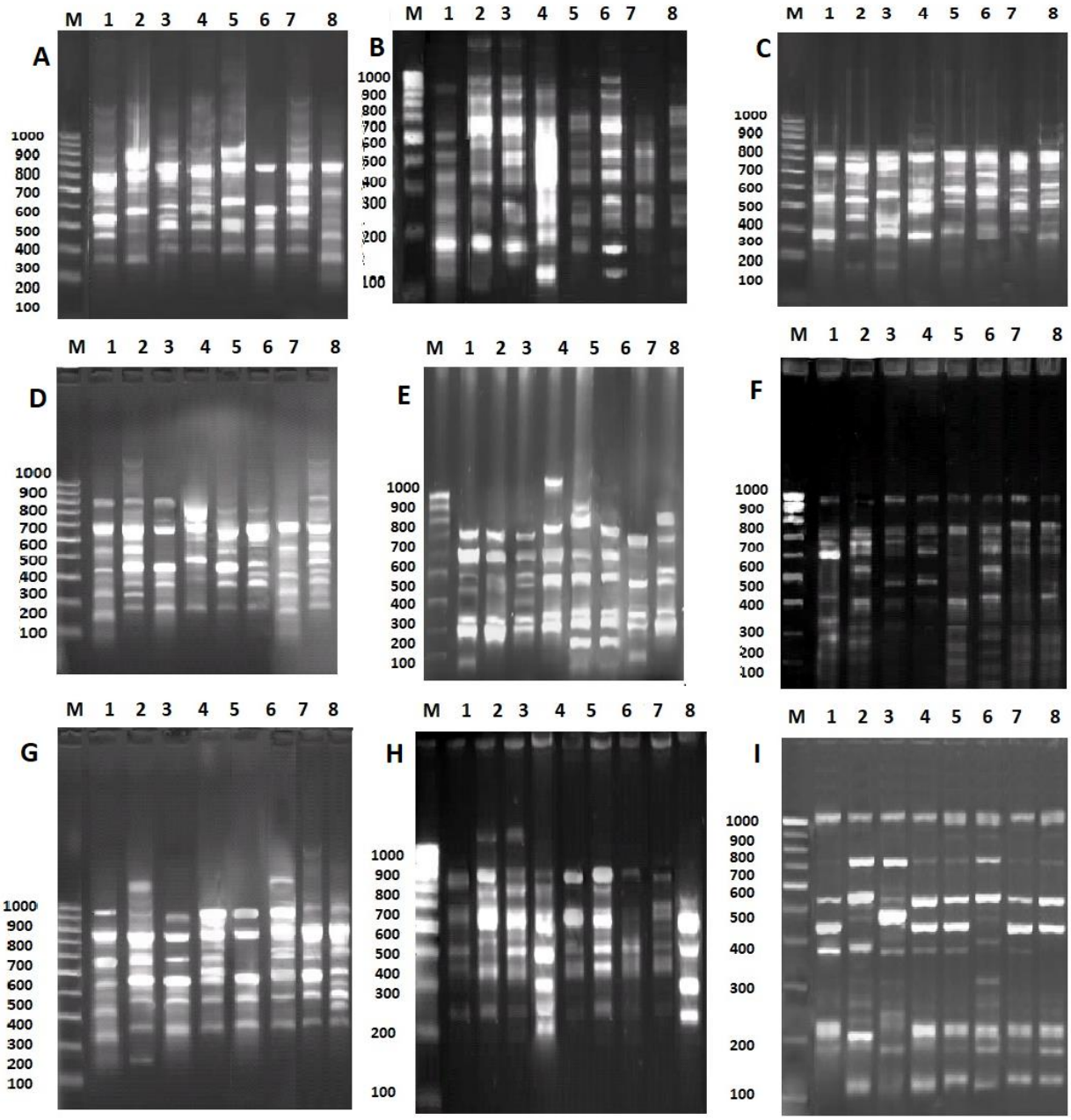

Fig. 2. ISSR-PCR using primer A: HB-09, B: HB-12, C: HB-14, D: UBC814, E: UBC840, F: UBC868, G: UBC835, H: HB-11 and I: HB-13. M: DNA marker. Lanes 1: FOT1, Lanes 2: FOT7, Lanes 3: FOT3, Lanes 4: FOT5, Lanes 5: FOT6, Lanes 6: FOT2, Lanes 7: FOT8 and Lanes 8: FOT4.

The dendrogram presented in Fig. (3) revealed that the eight tested isolates were divided into Three main clusters (A, B, and C). Cluster A is divided into two sub-clusters; sub-cluster A1 is split into two groups, group1 contains two subgroups, subgroup1 includes FOT6 isolate, and subgroup 2 includes FOT4, FOT3, and FOT5 isolates (FOT4 and FOT5 are highly virulent isolates), group2 contains FOT8 isolate. Sub-cluster A2 includes FOT6 isolate. However, cluster B includes the FOT1 isolate, while cluster C contains the FOT2 isolate.

Cluster analysis using ISSR markers showed that the isolates were distributed in

different groups, indicating that *F. oxysporum* has high genetic variability. These findings are in harmony with that detected by Bayraktar *et al*. (2008). They found that 74 isolates of *F. oxysporum* obtained from 13 regions in Turkey were divided into three major groups using ISSR, RAPD, and virulence analysis. ISSR and RAPD were used to classify *F. oxysporum f. melongenae* isolates into several groups, revealing high levels of genetic specificity and variability, implying that genetic variations are associated with the fungus in the Mediterranean region (Baysal *et al*., 2010). Cluster analyses assigned isolates of *F. oxysporum f. sp. carthami* from various Indian states to different clusters, reflecting a high level of genetic variations (Singh *et al*., 2019). Because *F. oxysporum* has no known sexual stage, the generation of new genetic recombinations does not result from meiotic crossing over (Booth, 1971; Gordon and Martyn, 1997). Instead, other mechanisms, including mutations, genetic drift, transposable elements, selection, gene flow, and hyphal anastomosis, are thought to drive species evolution (McDonald and Linde, 2002). Bayraktar *et al*. (2008) cleared that the low-genetic variation and high gene flow between populations influenced the *F. oxysporum f. sp. Ciceris* formation and evolution.

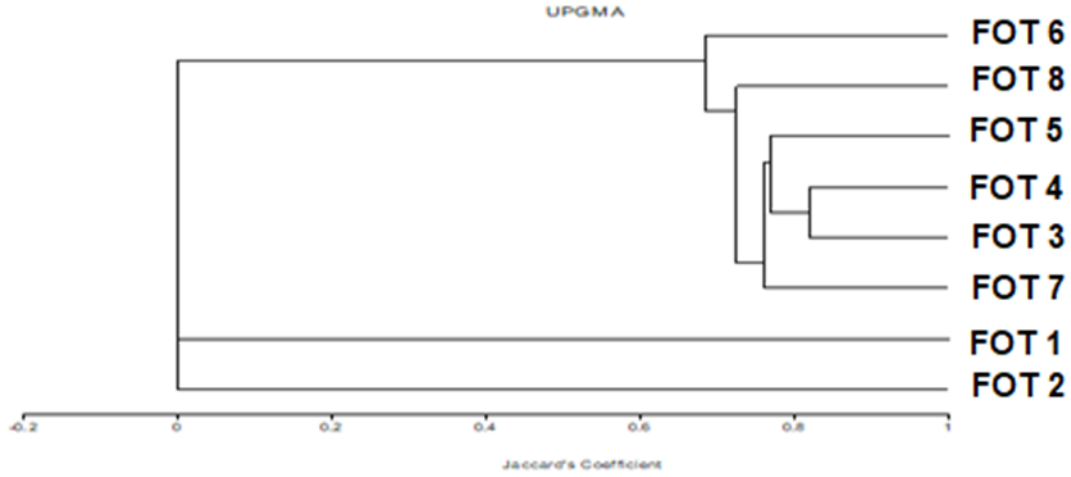

Fig. 3. The dendrogram shows the phylogenetic relationship among the eight *F. oxysporium* based on ISSRs analysis.

Table 4. A similarity matrix between the tested *F. oxysporum* isolates based on ISSR band pattern analysis and Jaccard index.

	FOT7	FOT1	FOT3	FO75	FOT6	FOT2	FOT8	FOT4
FOT7	100	64	68	64.8	68	70	67.4	64
FOT1		100	63.6	65	67	63.5	67.6	66
FOT3			100	79	79	64	76.4	82,6
FOT5				100	81	67.3	75	81.6
FOT6					100	70.8	72.3	78.5
FOT2						100	70.6	75
FOT8							100	76.6
FOT4								100

CONCLUSION

The findings of this study revealed a high level of variation between *F. oxysporum*, the causative agent of the tomato Fusarium wilt, using ISSR and virulence analysis. Both approaches were effective in analyzing the populations of *F. oxysporum* isolates. ISSR markers proved to be remarkably informative for studying the polymorphisms in *F. oxysporum* populations. One of the priority goals should be to use molecular markers to learn more about the molecular evolution of *F. oxysporum* that infects tomato plants in several Egyptian governorates, which is critical for breeding tomato against Fusarium wilt.

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الملخص العربى

التفريق بين عزلات الذبول الفيوزرايوم فى الطماطم باستخدام معلمات ISSR markers واختبارات المرضية

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يعتبر فطر فيوزاريوم أوكسيسبوروم يسبب الذبول الفيوزاريوم فى الطماطم ، وهو أحد الأمراض الفطرية الخطيرة التي تسبب خسائر كبيرة في إنتاج الطماطم في مصر. تم عزل ثماني عزلات من .F. oxysporum من نباتات طماطم مصابة جمعت من ست محافظات مصرية. كانت العزلات FOT4 و FOT2 و FOT5 شديدة المرضية، بينما كانت العزلات FOT6 و FOT8 متوسطة المرضية، وكانت FOT7و FOT3 و FOT1 ضعيفة المرضية. كانت جميع بادئات ISSRs المختبرة متعددة الأشكال وأنتجت 81 موقع DNA بأحجام جزيئية مختلفة. أظهر تحليل أنماط شظايا الحمض النووي 37 نطاقًا أحادي الشكل *monomorphic* و 44 حزمة متعدد الأشكال *polymorphism* ، مع تعدد أشكال 54.32٪ بين العزلات المختبرة. من بين العزلات المختبرة كان للبادئ UMC−840 أعلى مستوى من تعدد الأشكال (85.71٪) ، بينما كان للبادئ HB-13 أقل مستوى (33.33٪). تم تحليل أنماط Dendrogram و ISSR باستخدام طريقة UPGMA لاكتشاف العلاقة بين العزلات الثمانية. ولوحظ أعلى تماثل وراثي (82.9٪) بين عزلات FOT4 وFOT3 ، بينما لوحظ أقل تماثل وراثي (63.5٪) بين عزلات FOT1و .FOT2 . تم تصنيف العزلات الثمانية إلى ثلاث عناقيد رئيسية A و B و .C تتضمن العنقود A مجموعتين تحت عناقيد؛ تنقسم تحت العنقود الاول A1 إلى مجموعتين ، المجموعة 1 تتكون من مجموعتين فرعيتين، المجموعة الفرعية 1 تشمل العزلة FOT 6 ، والمجموعة الفرعية 2 تشمل العزلات FOT4 و FOT3 و FOT5 كانت العزلات (FOT 4 و FOT 5) كانت عالية الشدة المرضية ، المجموعة 2 بها عزلة .FOT8 تتضمن تحت العنقود A2العزلة FOT6 ، بينما يشتمل العنقود B على العزلة FOT1 ، وتتضمن العنقود C على العزلة FOT2 .