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AGGRESSIVENESS VARIATIONS AMONG ISOLATES OF *FUSARIUM OXYSPOIUM* F.SP. *LYCOPERSICI* BASED ON THE PATHOGENICITY TEST, HOST DEFENSE RESPONSE, AND ISOLATES GENETIC VARIABILITY

Sawsan S. EL-Shamy[1], Ibrahim A.A. Adss[2] and Alsayed M. Mashaheet[1]

[1]Department of plant pathology, Faculty of Agriculture, Damanhur University, El-Beheira, Egypt,

[2]Department of Genetics, Faculty of Agriculture, Damanhur University, El-Beheira, Egypt,

Corresponding author: Adssibrahim@agr.dmu.edu.eg

ABSTRACT

Fusarium oxysporum f.sp. lycopersici **(FOL) is a soilborne fungus and the causal agent of vascular wilt disease in tomato and results in severe yield losses, challenging tomato production in Egypt and worldwide. As a key step towards controlling this serious disease, this study investigates the aggressiveness of local FOL isolates, and their effects on the plant defense-related enzymes and gene expression, and to estimate the genetic variation among the isolates using RAPD-PCR in relation to their aggressiveness. Nine FOL isolates (Fo1 – Fo9) were isolated from diseased tomato field plants. Tomato (*Lycopersicon esculentum* Mill) cultivar 'loggain' was used to study the pathogenicity of the nine isolates and their effects on two**

defense-related enzymes: (1) Peroxidase (POD), (2) Polyphenol oxidase (PPO), and the gene expression of the two tested enzymes in the host was quantified using Real-time PCR. Three isolates showed different levels of aggressiveness on the tested cultivar (High: Fo5, followed by Fo3 and Fo1; Moderate: Fo8 and Fo7; Weak: Fo9 and Fo4). The highest enzyme activity and gene expression of PPO and POD were induced by isolates Fo5 and Fo3, followed by Fo1, Fo8, Fo7, and Fo2, because they caused moderate responses, while Isolates Fo9 and Fo4 isolates induced low enzyme activity and gene expression in the host. A high similarity (78.4%) was observed between Fo9 and Fo4 (included in subgroup 2 in the phylogenetic tree), followed by Fo2 and Fo9 with a similarity of 76.9%, while the similarity between Fo7 and Fo8 was 75.5% (included in group 1 in the phylogenetic tree). The lowest similarity (47.3%) was observed between Fo6 and Fo3. These findings showed differences in pathogenicity among the isolates and suggest a connection between the genetic variability and the aggressiveness of the various isolates.

Keywords: Defense-related enzymes. Pathogen, aggressiveness, phylogenetic, gene expression.

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INTRODUCTION

The modern world is beset with several issues related to food security. Traditional agriculture faces several challenges, including population growth, decreased land availability, pest and disease outbreaks, and drought. Among these, diseases and pests result in significant losses

(Bowyer, 1999). Plant pathogens seriously threaten the production of food, fiber, and bioproducts in the field and during storage, which makes them extremely important economically. The most effective way to handle these losses is to genetically modify plants **(Ashkani *et al.*, 2014)**.

Tomato (*Lycopersicon esculentum* Mill), is a significant horticultural crop in Egypt. This small herb, which belongs to the Solanaceae family, can be either an annual or a perennial crop. It suffers significant crop loss because of pathogenic fungi and bacteria. The most common of these are fungal infections, that cause significant damage to tomato fruits **(Chaurasia *et al.*, 2013)**.

Fungi-related plant diseases are a major global agricultural concern. Plant diseases are thought to cause total losses that amount to 25% of the yield in developed nations and nearly 50% in underdeveloped nations. Of these, fungal infections account for one-third of the cases **(Bowyer, 1999)**. The genus *Fusarium* includes some of the key pathogenic fungi to major crops worldwide. *Fusarium oxysporum* causes cortical rot and wilting diseases in over 100 plant species of significant economic importance **(Swift *et al.*, 2002)**. Crop rotation, greenhouse sanitation with chemicals or heat, regulating the processes of propagation material production and transfer, controlling seed infestation, the use of pathogen-suppressive growing media, biocontrol agents, fungicides, and soil and substrate sterilization with methyl bromide or solarization are some possible methods for managing diseases and controlling pathogens **(Gamliel and Yarden, 1998)**.

Species identification in the genus *Fusarium* is often based on both macro and microscopic characteristics. Nonetheless, most reports state that these characteristics are unstable. The most widely used techniques for identifying pathogens are morphological, biochemical, and allozyme characteristics; however, these methods rely on professional experience and could result in identification errors **(Szecsi and Dobrovolsky, 1985; Nelson *et al.*, 1983)**

Because this disease is persistent in tomato fields, the genetic variability of the local isolates of this serious pathogen must be evaluated. DNA marker-based molecular methods, like random amplified polymorphic DNA (RAPD) **(Belaj *et al.*, 2003; Williams *et al.*, 1990)**, as well as the amplified fragment length polymorphism (AFLP) **(Rotondi *et al.*, 2003)** represent cost-effective and efficient and methods to evaluate genetic diversity of fungi species from diverse groups, including *Agaricus blazei* **(Colauto *et al.*, 2002)**, *Alternaria* species **(Hong *et al.*, 2006)**, *Erythricium salmonicolor* **(Sebastianes *et al.*, 2007)**, Lactobacillus **(Torriani *et al.*, 2007)**, Metharizium anisopliae **(Freire *et al.*, 2001)**, and Trypanosoma **(Masiga *et al.*, 2006).**

Pathogen identification is currently conducted using genetic markers and nucleotide sequence analyses. DNA markers are currently used to analyze genetic variations with high genetic divergence and can identify multiloci in the genome **(Anne, 2006)**. In creating fungal genetic maps, molecular markers are frequently employed. For example, Cleaved Amplified Polymorphic Sequences (CAPs), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), and Inter Simple Sequence Repeats (ISSR).

The objectives of this study were 1) the isolation, purification, and identification of *FOL* local isolates, 2) the determination of the difference in aggressiveness between isolates, 3) the determination of the effect of difference in aggressiveness between isolates on the host defense enzyme and gene expression, and 4) the estimation of the genetic variation among the nine isolates using RAPD- PCR.

MATERIALS AND METHODS

The location of the study

This study was carried out in the laboratories and greenhouse, during 2022 and 2023 at the Faculty of Agriculture, Damanhour University. Each experiment was conducted three times.

Pathogen Isolation and Morphological identification

A total of 27 tomato root rot samples were gathered from tomato fields in Elbeheira Governorate, Egypt, and transported to the plant pathology lab where they were carefully cleaned with tap water and left to air dry. Tiny fragments of inner root tissue were extracted from areas close to the symptomatic root tissues. The fragments were surface sterilized for three minutes in a 3% solution of sodium hypochlorite (NaClO), then rinsed in distilled water, and placed between sterilized filter papers until they dried out. Subsequently, to avoid bacterial contamination, fragments were aseptically transferred to Petri plates with PDA containing 0.2 g/L of streptomycin sulfate, then they were incubated at 28^{o}C for five days **(Shaukat *et al.*, 2005; Bowyer, 1999)**. The isolated fungal cultures were purified using the hyphal-tip technique **(Brown, 1924)**. The isolated fungal cultures were then transferred to PDA slants and incubated at 28°C for one week. The pathogen identification was conducted according to **Gilman (1957)**, **Nelson *et al* (1983)**, and **Summerell *et al* (2003)** at the Agriculture Research Centre, Giza, Egypt, and carried out by the Department of Mycology and Plant Diseases, Institute of Plant Pathology Research.

Response of tomato cultivar 'loggain' to the infection with *Fusarium* isolates in greenhouse

Isolates Pathogenicity

Tomato cultivar 'Loggain' was used to study the pathogenicity of 9 isolates of the pathogenic fungus *Fusarium oxysporum* f. sp. *Lycopersici* (FOL). After five minutes of sterilizing with 1% NaClO, the host seeds were sawn into 20-cm plastic pots, each of which is filled with 3 kg of sterilized soil mixture of clay, peat moss, and sand (1:1:1, respectively). To prepare fungal inoculum, isolates of the pathogen were cultured for 10 days

at 25 ± 2°C in Erlenmeyer flasks of 250 ml, with Potato Dextrose Broth medium (PDB). The mycelia were removed from resultant cultures by filtration, then a hemocytometer was used to measure and adjust the conidial suspension concentration to 10 **(Szecsi *et al.*, 1985)** conidia per mL. When the host plants were 15 to 20 cm tall. To inoculate the soil, 50 milliliters of the conidial suspension (10^6 conidia/mL) were added to one kilogram of soil **(Elad and Baker, 1985)**.

Assessment of disease severity

The severity of FOL-induced wilting was scored using a 6-grades scoring scale according to **Liu *et al.* (1995)**, and was calculated using the equation:

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

Effect of the infection by different fusarium isolates on the activity of defense-related enzyme in tomato cultivars 'Loggain'

The differential responses of defense-related enzyme activity of peroxidase (POD) as well as the polyphenol oxidase (PPO) were determined using tomato cultivar 'loggain'. The samples were taken from the tissue surrounding the infection site. Root defense responses were assessed at 0, 1, 3, 9, and 15 days after inoculation (DAI). Each treatment was applied to five replicates. The data was statistically analyzed SAS 9.4 (SAS Inc., Cary, Noth Carolina, USA).

Assessment of peroxidase (POD) activity

Assessment of POD activity was conducted according to **Hammerschmidt and Kuc (1982)**. A pre-cooled (4°C) mortar and pestle were used to homogenize one gram of the root sample in two milliliters of 0.1M sodium phosphate buffer (pH 6.5). Then, the resultant homogenate was centrifuged for 15 minutes at 4°C at 10,000 rpm (Universal 32R,

Hettich Zentrifugen, Germany), whereas the supernatant functioned as the source of the enzyme. The reaction mixture, consisting of 1.5 mL of 0.05M pyrogallocallol, 0.5 mL of 1% hydrogen peroxide (H_2O_2) and 0.5 mL of enzyme extract, was incubated at 28±2°C. To begin the reaction of enzyme, the mixture's absorbance in the spectrophotometer was set to a wavelength of zero at 420 nm (Jenway, Model 6305, Bibby Scientific Limited, UK), then, records of the absorbance were taken for three minutes at 20-seconds interval. The control was a preparation of boiled enzymes. The changes in absorbance of the reaction mixture (min^{-1} g^{-1}) were used to express the peroxidase activity in the fresh tissue.

Estimation of polyphenol oxidase (PPO) activity

The activity PPO was measured according to **Mayer *et al*. (1965)**. For each root sample, one gram was homogenized in two milliliters of sodium phosphate buffer (0.1 M, pH 6.5) using in a pre-cooled mortar and pestle. After 15 minutes of centrifugation of the homogenates at 4°C at 10,000 rpm, the supernatant served as a source for the enzyme. To prepare reaction mixtures, the enzyme extract (200 µL) and sodium phosphate buffer (1.5 mL of 0.1 M, pH 6.5) were used. For the reaction initiation, 200 µL of catechol solution (0.01 M) was added to the reaction mixture before the incubation at room temperature. The absorbance was set to zero at 495 nm. PPO activity was recorded as the change in absorbance, measured in min^{-1} g^{-1} of the fresh tissue sample. The absorbance changes were recorded at 30-second intervals for two minutes.

POD and PPO gene expression quantification using Real-time PCR

Sample preparation

Samples of roots were collected from the host plants grown in the greenhouse at 0, 1, 3, 6, and 15 DAI to measure POD and PPO gene expression using Real-time PCR (RT-PCR).

Sterilization of glassware and plasticware with DEPC

To ensure glass and plasticware are free of RNases before being used to prepare and store RNA, they were treated with a water solution of diethylpyrocarbonate (DEPC) 0.1% (v/v) since RNase frequently contaminates them. Both glassware and plasticware were filled with DEPC and kept at 37°C for six hours, then repeatedly rinsed with sterile water. Next, it was heated to 100°C for fifteen minutes on a liquid cycle at one bar per square inch. Any DEPC residues that might otherwise carboxymethylate purine residues in RNA must have been eliminated by this process.

RNA isolation

The manufacturer's instructions were followed to extract the total RNA from tomato roots using the Guanidium isothiocyanate Method and BioTeke Corporation RNA Isolation kit I© (Maxim Biotech INC, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) of mRNA

The process that converts mRNA into complementary DNA (cDNA) without the reverse transcriptase and dNTPs is known as reverse transcription and is also referred to as the first-strand reaction. The reaction components were mixed with a DNA primer and a reverse transcriptase buffer and for one hour at 42°C. The RT-PCR's exponential amplification offers a very sensitive method for RNA molecules' detection at very low copy numbers.

Primer 5'-TTTTTTTTTTTTTTT-3' was used for the oligo (dT) reverse transcription reaction. A buffer containing $MgCl_2$, 2.5 µL (2.5 mM) dNTPs, 1 µL (10 pmol) primer, 2.5 µL RNA (2 mg/mL), and 0.5 unit of reverse transcriptase enzyme was prepared, and a 2.5 µL (5x) of the buffer were included in each 25 µL reaction mixture. The thermal cycler was set to run for one hour at 42°C and for ten minutes 72°C (enzyme killing), PCR

amplification was carried out. The result was kept at 4°C until it was needed.

Quantification of the PPO and POD gene expression using RT-PCR

Table (1) details the three specific primers developed by Pharmacia Biotech that were used in this study (Amersham Pharmacia Biotech., UK Limited, HP 79NA, England).

Table 1: Sequences and annealing temperatures of the Real-Time PCR's primers used in the study.

Primers		Primer sequence	Annealing (°C)
Peroxidase	F	GCTTTGTCAGGGGTTGTGAT	60
	R	TGCATCTCTAGCAACCAACG	
Polyphenol oxidase	F	CATGCTCTTGATGAGGCGTA	60
	R	CCATCTATGGAACGGGAAGA	
18S	F	5′-GTGCATGGCCGTTCTTAGTTG-3′	60
	R	5′-CAGGCTGAGGTCTCGTTCGT-3′	

The fermentase kit (Fermentas, Germany) was used to analyze the samples. Each reaction of 25 µL total volume consisted of 12.5 µL of 2x Quantitech SYBR® Green RT Mix, 1 µL of each of the 25 pm/µL forward and reverse primers, 1 µL of cDNA (50 ng), and 9.25 µL of RNase-free water. Before they were loaded into the wells of the rotor, all samples were spun. The RT-PCR program was approached as follows: the initial denaturation was done for ten minutes at 95°C; 40 denaturation cycles for 15 seconds at 95 °C; 30 seconds of annealing at 60°C; and 30 seconds of

extension at 72°C. Data collection was done in the extension phase. The reaction was carried out using the Rotor-Gene-6000 system (Qiagen, USA).

Real-time PCR data analysis

Utilizing the Rotor-Gene 6000 (Qiagen, ABI System, USA), the RT-PCR reaction was carried. According to **Livak and Schmittgen, (2001)**, ΔCq=Cq–reference gene, ΔΔCq=ΔCq – control, and ΔΔ C q expression = $2^{(-\Delta\Delta Cq)}$ were used to perform relative quantification of gene expression. The expression level of each of the target genes was normalized in relation to the 18S rRNA gene. The relative expression of the untreated (negative) control plants was set to 1 each time.

Fingerprinting of *Fusarium oxysporum* isolates using Random Amplified Polymorphic DNA (RAPD).

According to the producer's instructions, *FOL* genomic DNA fungus was extracted using G-spin TM Total DNA Extraction Kit. Isolates of *FOL* were distinguished and fingerprinted using PCR amplification using five RAPD primers **(Adss and Tabikha, 2021)**. PCR amplifications were conducted in a total volume of 25µL consists of 2.5 µL of 10x buffer, 2.5 µL of 50 mM $MgCl_2$, 2.5 µL of 4mM dNTPs,7 µL of 50 pmol primer, 1 µL of 10 ng *FOL* genomic DNA, and 0.2 µL (5 units/µL) of Taq DNA polymerase (Promega Germany). The PCR procedure was carried out using the following steps: initial denaturation for 5 min at 95 °C, 40 cycles at 95°C for 1 min, 1 min of annealing depending on primer temperature, extension for 1 min at 72°C, and lastly, an additional final extension step at 72°C for 10 min **(Istock *et al.,* 2001)**. Before loading each slot with 10 µL, the loading dye was added (2 µL). 100 volts was used in electrophorese, in 0.5 x TBE as the running buffer and 1.5% agarose/0.5x TBE gel. After staining gels with 0.5 µg/mL (w/v) ethidium bromide solution, the stain was removed with deionized water. At last, the gel was seen and recorded on camera with the help of the gel documentation

system. DNA bands were created using six primers, and the presence or absence of each primer's band was scored.

Table (2): Sequences of employed RAPD primers28 in the amplification of the genomic DNA.

Primer Code	Nucleotide Sequence 5\ ---------- 3\	Reference
OPC06	GAACGGACTC	Pourarian et al., 2018
OPC08	TGGACCGGTG	
OPP16	CCAAGCTGCC	
OPP19	GGGAAGGACA	
OPA16	AGCCAGCGAA	

Using RAPD-PCR DNA band patterns for dendrogram construction.

For each primer, the presence or absence of each of the amplified bands determined the score used in computer analysis of the data acquired by the band patterns of RAPD-PCR DNA. After excluding common bands, a band was designated as (1) if it was present in a fungal isolate, and when it was absent, it was designated as (0). According to Jaccard **(Jaccard, 1980),** Version 3.1 of the Multi-Variate Statistical Package (MVSP) was utilized in generating similarity coefficients through pairwise comparison between the fungal isolates depending on the presence or absence of unique and shared polymorphic bands. The dendrogram was constructed using UPGMA (Unweighted pair-group Method with Arithmetical Averages) based on the similarity coefficients.

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RESULTS

Isolation and identification of the *Fusarium oxysporum* associated with tomato wilt

Nine isolates (Fo1, Fo2, Fo3, Fo4, Fo5, Fo6, Fo7, Fo8, and Fo9) of *Fusarium oxysporum* f. sp. *lycopersici* (*FOL*) of tomato Fusarium wilt were obtained from infected tomatoes collected from El-Beheira governorate.

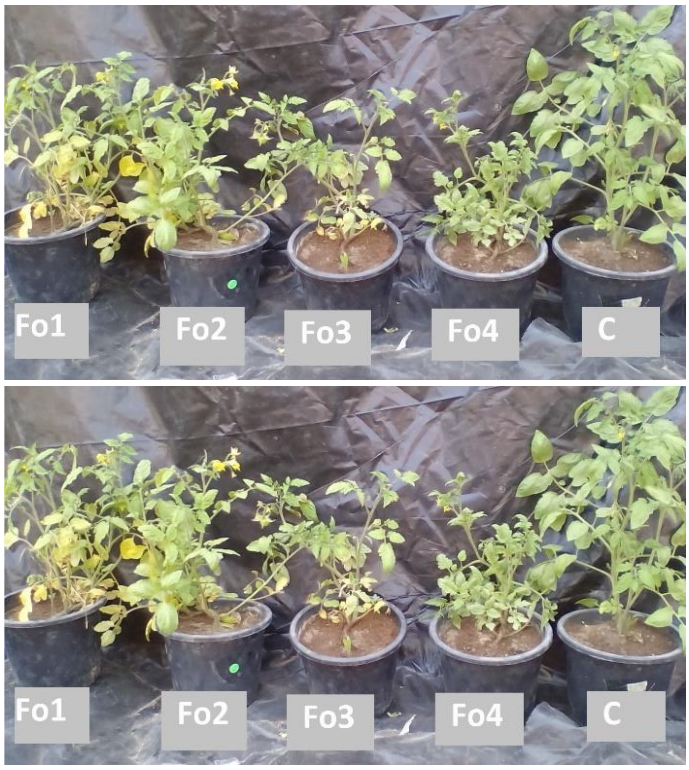

Fig. 1: Pathological test of tomato cultivar 'Loggain' in response to the infection with nine isolates of *Fusarium oxysporum* f. sp. *Lycopersici.*

Pathogenicity test

The *FOL's* pathogenicity tests for the 9 isolates were made using tomato cultivar Loggain. All isolates were virulent and differed in their aggressiveness according to the data in Table (3) and Fig. (1); the isolates Fo5 was the most aggressive, causing 67% disease severity, followed by Fo3 and Fo1, which caused 59% and 55% disease severity, respectively; Fo8 and Fo7 were moderately aggressive causing 47% and 45% disease severity, respectively; F02 and F06 resulted in 37% and 33% disease severity, respectively. The isolate F09 was weakly aggressive, producing 20%, and isolate Fo4 was the least aggressive, producing 8% disease severity.

Table 3: Disease severity on tomato cultivar 'Loggain' because of the infections with nine *Fusarium oxysporum f. sp. lycopersici* Isolates.

***Fusarium* isolates**	**Diseased severity %**
Fo5	67^{a*}
Fo3	59^{b}
Fo1	55^{b}
Fo8	47^{c}
Fo7	45^{c}
Fo2	37^{d}
Fo6	33^{d}
Fo9	20^{e}
Fo4	8^{f}

*Data are the average of three replicates. $LSD_{0.05} = 5.13$

The differential enzyme activity of defense-related in response to the infection with different fusarium isolates with different levels of aggressiveness

The nine isolates of Fusarium with varying aggressiveness that were tested in the pathogenicity test (Table 3) were utilized to assess the activity of peroxidase (POD) and polyphenol oxidase (PPO) as two major defense-related enzymes. Tomato roots were inoculated with fusarium isolates and the defense responses were measured at 0, 1, 6, 9, and 15 days after inoculation.

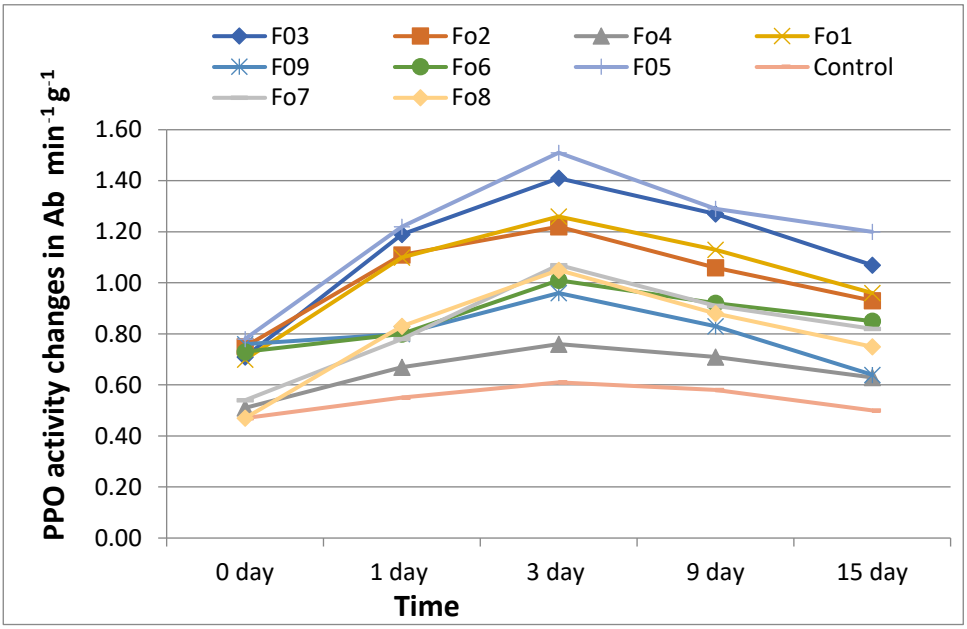

Fig. 2: Polyphenol oxidase (PPO) activity in tomato cultivar 'Loggain' after being infected with nine *Fusarium oxysporum* f. sp. *lycopersici* isolates.

Data in Fig. (2 and 3) show PPO and POD activity, respectively. The highest level of PPO and POD enzyme activity was recorded in tomato cultivar 'Loggain' as a result of the infection with Fo5 and Fo3 isolates compared to the control in most of the time after infection (these isolates were highly aggressive in pathogenicity test), followed by Fo1, Fo8, Fo7

and Fo2 isolates as they induced moderate levels of PPO and POD activity compared to the control in most of the time after infection (these isolate were moderately aggressive in the pathogenicity test), while the Fo9 and Fo4 isolates recorded low PPO and POD activity compared to the control in the most of the time after infection (these isolate were a less aggressive in pathogenicity test).

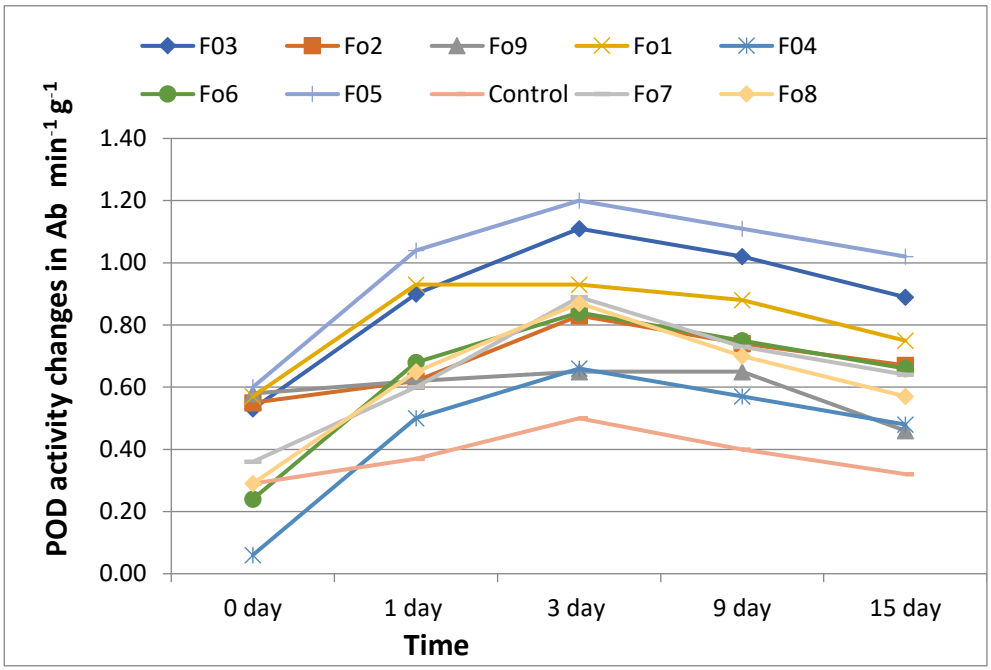

Fig. 3: Peroxidase (POD) activity in tomato cultivar 'Loggain' after being infected with nine *Fusarium oxysporum* f. sp. *lycopersici* isolates.

Determination of the varying responses of defense PPO and POD gene expression to the infection with different fusarium isolates with different levels of aggressiveness

Data presented in Figs (4 and 5) revealed the different levels of gene expression of POD and PPO in tomato cultivar 'Loggain' among the host plants infected with the nine isolates compared to the control. In most of the extended period of infection, the Fo5 and Fo3 isolates showed high levels of PPO and POD gene expression when compared to the control (these isolates were highly aggressive in the pathogenicity test and induced high defense-related enzyme activities). The Fo1, Fo2, Fo7, and Fo8 isolates showed moderate levels of PPO and POD gene expression when compared to the control (these isolates were moderately aggressive in the pathogenicity test and induced moderate defense-related enzyme activities), whereas Fo9 and Fo4 isolates showed low levels of PPO and POD gene expression when compared to the control in most of the time after infection (these isolates were the least aggressive in pathogenicity test and induced low defense-related enzyme activities).

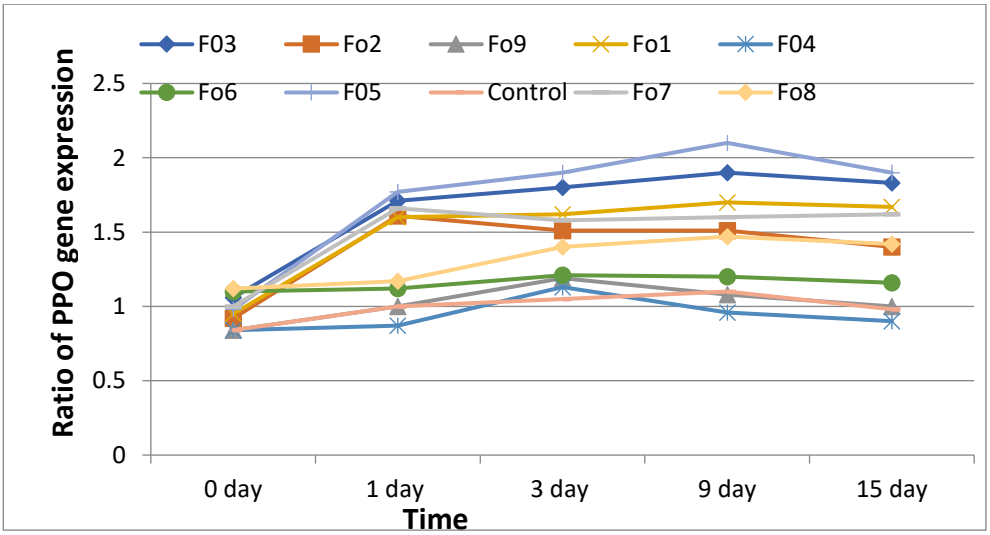

Fig. 4: Polyphenol oxidase (PPO) gene expression in tomato cultivar 'Loggain' after being infected with nine isolates of *Fusarium oxysporum* f. sp. *lycopersici*.

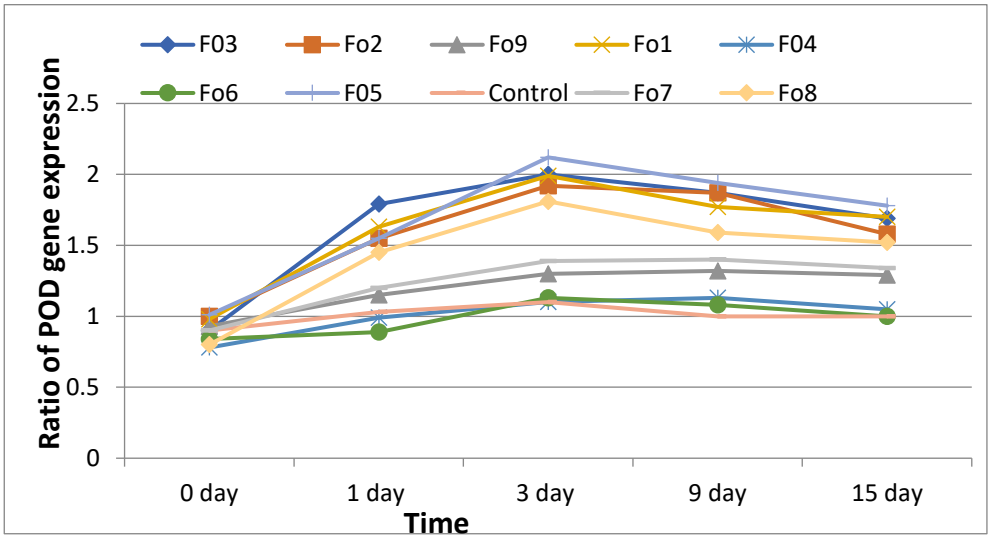

Fig. 5: Peroxidase (POD) gene expression in tomato cultivar 'Loggain' after being infected with nine isolates of *Fusarium oxysporum* f. sp. *lycopersici*.

Isolates fingerprinting using Inter Simple Sequence Repeat.

Five random decamer oligonucleotide primers were used for RAPD analysis to investigate the genetic diversity between nine *F. oxysporium* isolates. As shown in Fig. (6), the amplification profiles of the primers OPC06, OPC08, OPP16, OPP19, and OPA16 showed polymorphic patterns among the tested isolates. The highest similarity was observed between isolate Fo9 and Fo4 (78.4%), followed by Fo2 and Fo9 isolates (76.9%), while the similarity between Fo7 and Fo8 was 75.5%. The lowest similarity was observed between Fo6 and Fo3 isolates was 47.3% (Table 4).

Cluster analysis of RAPD results

UPGMA method was used to analyze the RAPD band patterns to find the relationship between the nine examined isolates by constructing the similarity matrix needed for generating a dendrogram. The computer analysis relied only upon the presence or absence of the DNA bands. The resulting dendrogram displays the linkage distance. The isolates under examination were categorized as two primary clusters (A and B) as shown in Figure Fig. (7). Cluster A included two sub-clusters, (sub-cluster A1 and sub-cluster A2). There are two groups in sub-cluster A1, group 1, which include two subgroups: subgroup 1 includes Fo2 isolate, and subgroup 2 includes Fo4 and Fo9 (these isolates were weakly aggressive in pathogenicity test and induced enzyme activity); group2 includes Fo3 and Fo5 (these isolates were highly aggressive in pathogenicity test and induced enzyme activity). Sub-cluster A2 consists of two groups: group 1 includes Fo8 and Fo7 (these isolates were moderately aggressive in the pathogenicity test and induced enzyme activity); group 2 includes Fo1 isolate.

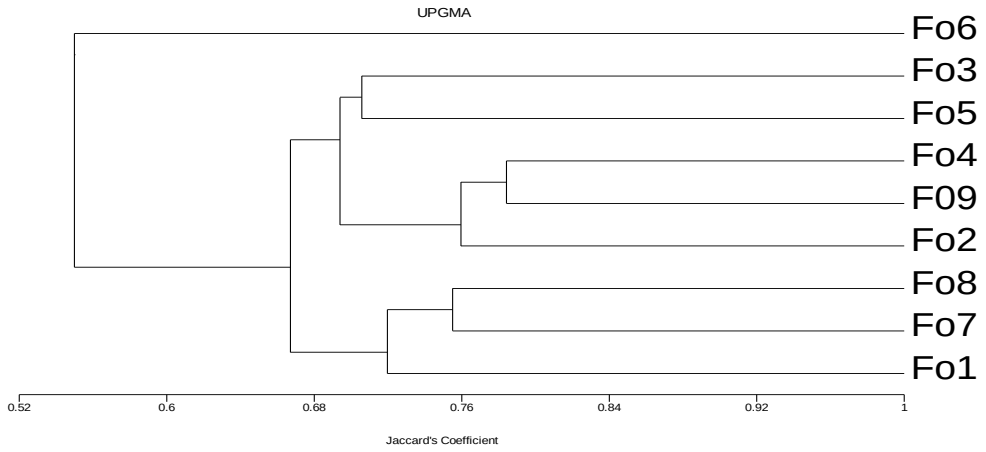

Fig. 7. Dendrogram showing phylogenetic relationship among nine isolates of *Fusarium oxysporum* f. sp. *lycopersici* as concluded from RAPD-DNA results

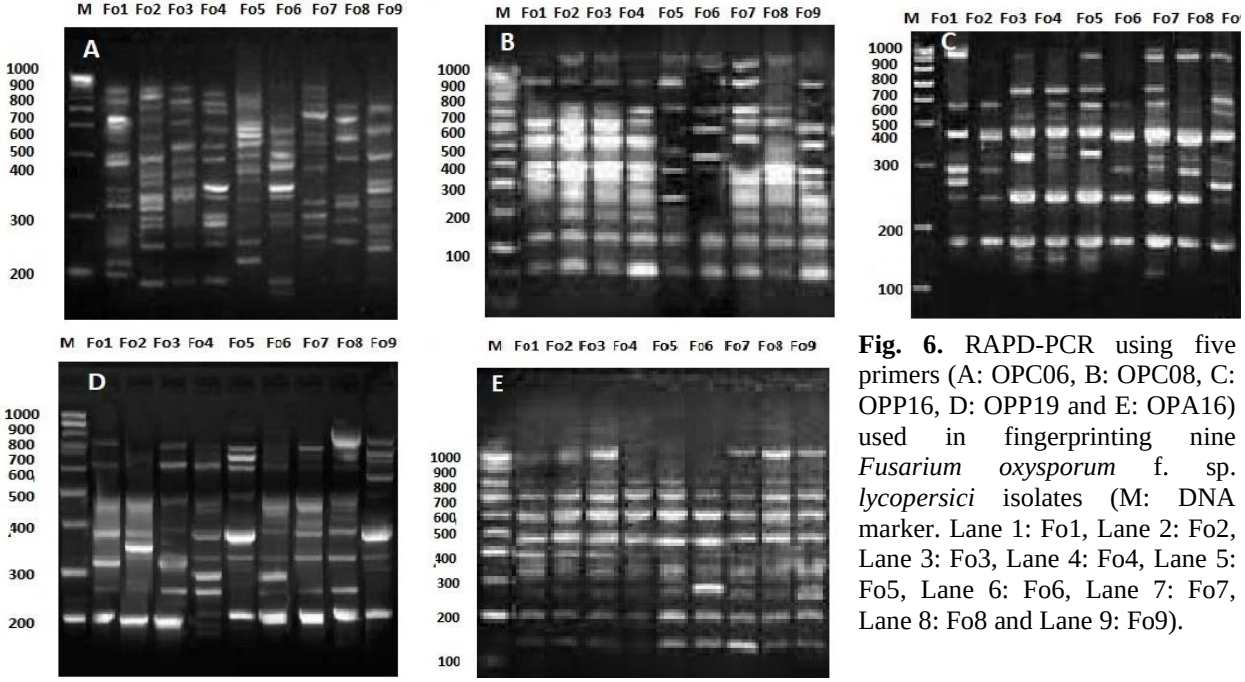

Fig. 6. RAPD-PCR using five primers (A: OPC06, B: OPC08, C: OPP16, D: OPP19 and E: OPA16) used in fingerprinting nine *Fusarium oxysporum* f. sp. *lycopersici* isolates (M: DNA marker. Lane 1: Fo1, Lane 2: Fo2, Lane 3: Fo3, Lane 4: Fo4, Lane 5: Fo5, Lane 6: Fo6, Lane 7: Fo7, Lane 8: Fo8 and Lane 9: Fo9).

Table 4. Jaccard's genetic Similarity matrix based on the analysis of RAPD-DNA of nine isolates of *Fusarium oxysporum* f. sp. *lycopersici*.

	Fo1	Fo2	F09	Fo4	Fo5	Fo6	Fo7	Fo8	Fo3
Fo1	**1**								
Fo2	**0.727**	**1**							
F09	**0.696**	**0.769**	**1**						
Fo4	**0.679**	**0.75**	**0.784**	**1**					
Fo5	**0.661**	**0.636**	**0.765**	**0.712**	**1**				
Fo6	**0.554**	**0.585**	**0.527**	**0.627**	**0.519**	**1**			
Fo7	**0.722**	**0.698**	**0.698**	**0.648**	**0.66**	**0.577**	**1**		
Fo8	**0.717**	**0.725**	**0.692**	**0.642**	**0.623**	**0.538**	**0.755**	**1**	
Fo3	**0.673**	**0.648**	**0.712**	**0.692**	**0.706**	**0.473**	**0.582**	**0.604**	**1**

DISSCUSIONS

Tomato fusarium wilt is among the most serious tomato diseases, caused by the disease agent *Fusarium oxysporum sp. Lycopersici* (*FOL*). Particularly in indoor environments with high temperatures and humidity, it results in considerable damage and financially devastating crop losses **(Agrios, 2005)**. The main objectives of this study were to isolate, purify and identify different isolates of FOL from El-Beheira Governorate, Egypt, and to determine the difference in aggressiveness among isolates, as well as to determine the effect of the isolates' differential aggressiveness on the induction of host plant's defense enzyme activity and gene expression and to estimate the genetic variation among the isolates using RAPD- PCR.

In this study, nine isolates of FOL were collected from wilted tomato plants grown in the fields of El-Beheira Governorate (Fo1, Fo2,

Fo3, Fo4, Fo5, Fo6, Fo7, Fo8 and Fo9). The diversity among the pathogen's population is in line with the findings of **Abou-Zeid *et al* (2016)**, who reported that six distinct pathogenic isolates of FOL were obtained from the Egyptian governorates of Dakahlya and Gharbya, two neighboring governorates to El-beherira. Several researchers have also reported the presence of *FOL* as the pathogenic causal agent of tomato wilt rot **(Abou-Zeid *et al.*, 2016)**. All isolates tested in this study were virulent and differed in aggressiveness, ranging from highly aggressive to moderate and weakly aggressive in this study. These findings also agree with those published by **Abou-Zeid *et al.* (2016)**, who reported that the six distinct pathogenic FOL isolates obtained from Dakahlya and Gharbya also differed in aggressiveness, as the FOL isolate F3 from collected form Dakahlya was more aggressive and resulted in a 96.67% wilt severity, which was significantly higher than the 71.67% produced by Tanta isolate F4. Variations in aggressiveness were also observed among local isolates of *Fusarium graminearum* isolated from Argentina **(Malbrán *et al.*, 2012).**

Following the observation of aggressiveness variations among the tested FOL isolates, their effects on the host responses and the isolates' ability to cause the disease were investigated using defense enzyme activity and defense genes expression. Because the nine isolates differed in their aggressiveness, they led to different levels of PPO and POD enzyme activity for each isolate. The isolates that recorded high aggressiveness in the pathogenicity test showed the highest level of enzymes activity and gene expression in in the host, such as Fo5 and Fo3 and while the isolates that were moderately aggressive in the pathogenicity test recorded moderate levels of enzyme activity and gene expression in the host, such as Fo1, Fo8, Fo7 and Fo2, whereas the least aggressive isolates also recorded low level of enzyme activity and gene expression in host such as Fo9 and Fo4 isolates. These findings echo the results obtained by **Malbrán *et al*. (2012),** who studied the variations in the aggressiveness of *F. graminearum* isolates from Argentina up on point-inoculating the spikes of wheat plants grown in the field.

Even in cases where morphological descriptions are severely restricted, genetic markers can be used to detect similarities and differences among various isolates of the same species. Among these, RAPD can generate multilocus profiles that span the entire genome despite the lack of previous genetic or sequence data, in spite of some disadvantages such as the dominant nature and stringent optimization of the assay. This study used a RAPD marker-based molecular profiling technique that can identify genetic diversity among the nine isolates of FOL. Significant genetic variations among the FOL isolates were detected as reflected in numerous polymorphic markers. The Fo9 and Fo4 isolates were weakly aggressive in pathogenicity test and enzyme activities, these isolates recorded the highest similarity observed in the phylogenetic tree in the same subgroup 2.

The isolates Fo3 and Fo5 were highly aggressive in the pathogenicity test and enzyme activities, and they are included in group 2 in phylogenetic tree, while the isolates Fo8 and Fo7 were moderately aggressive in the pathogenicity test and enzyme activities and were included in group 1 in phylogenetic tree. RAPD technique was utilized in several pathosystems to assess genetic diversity. For example, it has been used to study populations of *Alternaria solani* from Brazil and the results divided the isolates into two large clusters (**Lourenço et al., 2009)**, and it was similarly used to study *Alternaria alternata* isolates (**Guo et al., 2004)**. These findings of this study also agreed with the results obtained by **Elagamey *et al*. (2020)**, who observed a significant level of genetic diversity when used the identified polymorphisms in RAPD and protein profiles, and their cluster analysis matched the pathogenicity results. Previously, it was once thought that pathogen isolates differ in their pathology and morphology due to different collection regions, and that as long as isolates were taken from the same location, genetic variation could not exist between them. According to the findings of the current investigation, the observed genetic diversity of the investigated FOL isolates demonstrates that different genetic variations among the fungus isolates could arise in the same geographic area.

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CONCLUSIONS

The findings of this study support the hypothesis that there is a connection between the genetic variability and the aggressiveness of various isolates of FOL, as evidenced by the close genetic similarity among isolates with each of the different aggressiveness groups. The more aggressive the isolate is, the more enzymatic defenses the plant secretes, and vice versa. This variation in isolate-induced host response is reflected in both gene expression and enzymatic defense secretion. Genetic variations can occur among FOL isolates even though they were collected from the same geographical location.

Conflict of interests

The authors hereby declare no conflict of interests.

Significance statement

This study shows that nine isolates of *Fusarium oxysporum sp. Lycopersici* (FOL), the pathogenic causal agent of tomato wilt, differ in their pathogenicity and suggests a connection between the genetic variability and the aggressiveness of the various isolates. The more aggressive the isolate is, the more gene expression and enzymatic defense secretion the host plant secretes, and vice versa. Genetic variations among FOL isolates can occur even in isolates collected from the same geographical location.

Author's contribution

El Shamy, S. carried out the plant pathology experiments and contributed to the data analysis and interpretation of the results, and the writing of the manuscript. **Adss, I.A.** carried out the genetics experiments and contributed to the genetic data analysis, the interpretation of the results, and the writing and revision of the manuscript. **Mashaheet, A.M.**

contributed to the data analysis and interpretation of the results, the writing and revision of the manuscript.

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اختلافات الشراسة المرضية بين عزلات Fusarium oxyspoium f.sp. lycopersici بناءً على اختبار القدرة المرضية واستجابة دفاع العائل والتنوع الجيني للعزلات

سوسن صلاح الدين الشامي[1] و ابراهيم احمد عدس[2] و السيد سعد مشاحيت[1]
[1]قسم أمراض النبات، كلية الزراعة، جامعة دمنهور، البحيرة، مصر،
[2]قسم الوراثة، كلية الزراعة، جامعة دمنهور، البحيرة، مصر،

الملخص

الخلفية والأهداف: Fusarium oxysporum f.sp. lycopersici (FOL) :هو فطر ينتقل عن طريق التربة وهو العامل المسبب لمرض ذبول الأوعية الدموية في الطماطم ويؤدي إلى خسائر فادحة في المحصول، مما يشكل تحديًا لإنتاج الطماطم في مصر والعالم. كخطوة أساسية نحو السيطرة على هذا المرض الخطير، تبحث هذه الدراسة في عدوانية عزلات الفيوزاريوم FOLالمحلية، وتأثيراتها على الإنزيمات المرتبطة بدفاع النبات والتعبير الجيني، وتقدير التباين الجيني بين العزلات باستخدام RAPD-PCR فيما يتعلق بعدوانيتها. **المواد والطرق:** تم عزل تسع **عزلات** (Fo1 – Fo9) FOL من نباتات الطماطم المصابة. تم استخدام صنف الطماطم 'loggain' لدراسة مسببات الأمراض للعزلات التسع وتأثيراتها على إنزيمين مرتبطين بالدفاع: وهما بيروكسيديز **(POD)**، و بوليفينول أوكسيديز **(PPO)** ، وتم تحديد كمية التعبير الجيني للإنزيمات المختبرة في العائل باستخدام تفاعل البوليميراز المتسلسل في الوقت الحقيقي. **النتائج**: أظهرت ثلاث عزلات مستويات مختلفة من العدوانية على الصنف المختبر (العزلات العالية كانت Fo5،**و** يليها Fo3 **و**Fo1؛بينما كانت المعتدلة Fo8 **و**Fo7؛ بينما كانت الضعيفة Fo9 **و** Fo4**)** و كان أعلى نشاط إنزيمي وتعبير جيني لـ PPO **و** POD بواسطة العزلات Fo5 **و**Fo3، تليها Fo1**و** Fo8**و** Fo7**و**Fo2، لأنها تسببت في استجابات معتدلة، في حين أن العزلات Fo9 **و** Fo4 حفزت نشاط إنزيمي وتعبير جيني منخفض في العائل. لوحظ تشابه كبير (78.4%) بين Fo9 **و** Fo4(المدرجين في المجموعة الفرعية 2 في الشجرة التطورية)، يليهما Fo2 **و** Fo9 بنسبة تشابه 76.9%، بينما كان التشابه بين Fo7 **و**Fo8 75.5% (المدرجين في المجموعة 1 في الشجرة التطورية). ولوحظ أدنى تشابه (47.3%) بين Fo6 و.Fo3 **الخلاصة:** أظهرت هذه النتائج اختلافات في القدرة المرضية بين العزلات وتشير إلى وجود صلة بين التباين الوراثي وعدوانية العزلات المختلفة.