H

EGYPTIAN ACADEMIC JOURNAL OF
BIOLOGICAL SCIENCES
BOTANY

ISSN 2090-3812

www.eajbs.com

Vol. 13 No.2 (2022)

Citation: Egypt. *Acad. J. Biolog. Sci.* (H. Botany) *Vol.13(2) pp275-287(2022)*
DOI: 10.21608/EAJBSH.2022.289358

Egypt. Acad. Journal Biology. Sci., 13(2):275-287(2022)

Egyptian Academic Journal of Biological Sciences
H. Botany
ISSN 2090-3812
www.eajbsh.journals.ekb.eg.

Pathological and Molecular Variability *of Fusarium oxysporum* f.sp *lycopersici* Infecting Tomato in Egypt.

Mahmoud A. Gaber*, Ahmed F. El-Bebany*, Magda M. Fahmy, Sayed S. Aboshosha and Elsayed E. Wagih

Department of Plant Pathology, College of Agriculture, El-Shatby, University of Alexandria, Alexandria, Egypt

*E-mail: mahmoudgaber@alexu.edu.eg - aelbebany@yahoo.com

ARTICLE INFO

Article History
Received:21/10/2022
Accepted:20/12/2022
Available:25/12/2022

***Keywords*:** Tomato- *Fusarium oxysporum*- ITS- pathogenicity test - Egypt.

ABSTRACT

Tomato wilt disease is one of the most destructive fungal diseases infecting tomato plants in Egypt. The disease is caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL), a notorious pathogen that colonises xylem vessels of its host causing wilt besides other disease-associated symptoms. In total, eighteen different isolates of FOL were recovered from specimens collected from characteristic symptom-expressing tomato plants growing in five different governorates of Egypt, namely, Alexandria, El-Beheira, Kafr El-Sheikh, Giza and Assiut. Of these isolates, 13 were obtained from the root, 4 from the rhizosphere and one from the stem. Isolates were identified based on their cultural and microscopic morphological features. The pathogenicity of the eighteen isolates was tested against four different commercial tomato varieties (030, Dussehra, 86, and 111) in Egypt. Two isolates (FOL4013 and FOL4014) from Giza showed high virulence, while three isolates (FOL3010, FOL3011 and FOL3112) from Kafr El-Sheikh exhibited low virulence. The isolates from El-Beheira and Assiut showed intermediate virulence grades across the spectrum. The radial growth rate of the mycelium and conidia sporulation were assessed for all isolates. Radial growth rate ranged from 0.6 to 0.9 cm/24h with conidia sporulation rate ranging from 1.2 to 2 spore per 10 cm^3 ($x10^6$) of the suspension. The variation in rates among different strains was statistically significant at $P \geq 0.05$. Five of the studied isolates were molecularly characterised using PCR amplification of the ITS region followed by DNA sequencing to identify their genetic relationship and the corresponding phylogenetic tree was graphically presented.

INTRODUCTION

Tomato (*Solanum lycopersicum L.*) is a popular vegetable crop grown for its edible fruit, which is rich in vitamins and minerals. Tomatoes are used in a wide variety of dishes and are also a popular ingredient in sauces and condiments (Erba *et al.*, 2013). The tomato crop is also considered one of the strategic crops in Egypt and worldwide, for both consumers and industry. Henceforth, it has been the focus of research studies, breeding programmes and crop improvement strategies for many years (Quinet *et al.*, 2019). Tomato is a warm-season crop that grows in a wide range of climatic conditions worldwide.

Tomato is one of the essential growing vegetable crops in Egypt with a production value of 7.9 million tons in 2016 making Egypt one of the largest tomato producers in the world (FAO, 2019). In 2015, The tomato cultivated area in Egypt accounted for around 32% of the total vegetable cultivated area, making the tomato the most cultivated vegetable in Egypt (FAO, 2019). However, tomato plants are highly susceptible to many diseases of which Fusarium wilt is considered to be one of the most important diseases affecting their productivity (Khlode *et al.,* 2016b). *Fusarium oxysporum* f. sp. *Lycopersici* (FOL)-infected tomato plants show wilt symptoms associated with old leaf chlorosis due to severe damage to the xylem tissue that affects plant water and nutritional intake (McGovern 2015).

The pathogen, *Fusarium oxysporum*, is a large and complex species, which contains many formae speciales (f.sp.) that infect different host plants. The f.sp. lycopersici is specific to tomato plants and was first described by Saccardo in 1892 (Khlode *et al*., 2016b).

Fusarium oxysporum f. sp. *Lycopersici* is a facultative saprophyte that can be cultured on potato dextrose agar (PDA) or potato sucrose agar (PSA) at 25°C for 7-14 days where its colonies usually appear as white to light-gray, cottony and fluffy in texture.

Under the microscope, FOL hyphae are hyaline and septate, with a smooth surface. Microconidia are cylindrical and smooth-walled and measure 2-4 x 6-8µm. Macroconidia are fusiform, three-septate, and measure 15-35 x 2-4 µm. Chlamydospores are round and thick-walled (Manikandan *et al.,* 2018; Srinivas *et al*. 2019).

The fungus can be identified and characterised through various molecular techniques. One method of identification is by using polymerase chain reaction (PCR) amplification of specific genetic markers, such as the translation elongation factor 1-alpha (EF-1α) gene and internal transcribed spacer (ITS). This technique can accurately identify FOL from other closely related Fusarium species (O'Donnell *et al*. 2000; Singha *et al*. 2016).

The disease leads to a significant loss in tomato yield every year in Egypt and the rest of the whole world, thus making Fusarium infection one of the prominent limiting factors in tomato cultivation areas. For this, the need to develop and evaluate new bio-control agent(s) to control the Fusarium wilt of tomatoes has become an indispensable approach in Egypt. For this, several biological and chemical elicitors have been studied with the aim to fight Fusarium, and their effect was evaluated via gene expression analysis of plant defense genes and proteomic analysis (Chakraborty, *et al.,* 2017; Jaiswal *et al.,* 2020; Hahn, 1996; Ramamoorthy *et al.,* 2002)

The objectives of this study were, therefore, to diagnose tomato wilt disease in different tomato growing locations in Egypt and to identify the soil-borne causal fungus (FOL) using both conventional and molecular techniques, and to assess the virulence of the obtained isolates on some selected commercial tomato cultivars.

MATERIALS AND METHODS

1. Collection of Diseased Tomato Specimens:

Tomato plants displaying disease wilt symptoms along with other associated symptoms characteristic to FOL-infected tomatoes were collected from five different governorates of Egypt, namely, Alexandria, El-Beheira, Kafr El-Sheikh, Giza and Assuit during the year 2021. Collected specimens were used for the isolation of the causal agent. The specimens were separated into roots, stems and rhizosphere.

2. Isolation, Purification, And Preservation of The Causal Pathogen:

Collected specimens were thoroughly washed with tap water and small pieces from the infected areas were cut to 0.5 cm. They were then sterilised by being immersed in a 1% (W/V) sodium hypochlorite (NaOCl) solution for 30 seconds, followed by multiple rinses in sterilised distilled water to remove excess hypochlorite. Samples were dried using sterilised filter paper and plated in 9-cm diameter Petri plates with potato dextrose agar (PDA) medium

(200g potato extract, 20g dextrose and 20 g agar per litre). Plates were incubated at 23°C for 7–10 days with frequent monitoring for the development of typical white mycelial growth of *Fusarium oxysporum* and subcultured onto fresh PDA medium in plates.

3. Morphological Characteristics:

Isolates were observed for cultural characters and morphology of conidia. Colonies exhibiting the taxonomic features of *Fusarium oxysporum* were identified according to Khlode *et al.,*2016a. Morphological identification was based on characteristics of the macroconidia, microconidia, chlamydospores and colony growth traits.

4. Pathogenicity Test:

4. 1. Root Dip Technique:

Tomato inoculation using root dip technique the 18 FOL isolates were grown on (PD) for 14 days. Conidiospores suspensions 10^6 spore/ml were prepared by scratching the surface of the active growing mycelium in sterile distilled water. The conidiospore suspension concentration was adjusted using a Haemocytometer count plate.

The virulence of various strains was studied by exposing them to four different tomato cultivars. A total of 18 strains of FOL were examined for their pathogenic potential. Healthy tomato seedlings that were 25 days old were inoculated using the standard root dip method (as described by (Gonzalez-Cendales *et al.,* 2016)). The seedlings were gently removed from the soil, thoroughly rinsed to remove any dirt, and had the tips of their roots trimmed to approximately 1 cm. These trimmed roots were then immersed in the conidial 10^6 suspensions of each strain for 30 minutes. As a control, seedlings were subjected to the same procedure but were dipped in sterile water instead of the conidial suspension. The inoculated seedlings and the controls were then transferred to mini pots (15 cm in diameter) filled with a mixture of sterilized soil and sand (in a 1:1 ratio), with one seedling per pot and three replicates for each isolate. The seedlings were grown in a greenhouse with day and night temperatures ranging from 25 to 30°C. They were watered daily and given a single dose of NPK fertilizer (20:20:20). Symptoms of the disease began to appear 15 to 20 days after the artificial inoculation.

4. 2. Disease Index:

Disease severity was assessed on tomato plants from 2 weeks of FOL inoculation up to 40 days according to (Fakhouri and Buchenauer 2003; Song *et al.,* 2004). The severity of foliar yellowing was evaluated by assigning each leaf a rating between 0 and 4 based on the extent of yellowing and wilting symptoms. The average rating was then calculated for the entire plant using the formula: % of foliar yellowing = (Sum of foliar yellowing values / (4 x Total number of leaves)) x 100. In the current study, the following grading system was used: 0 = Healthy plants; 1 = Slight chlorosis, wilting, or stunting in less than 25% of the plant leaflets; 2 = Moderate chlorosis, wilting, or stunting in 25% to 50% of the plant leaflets; 3 = Severe chlorosis, wilting, or stunting in 50% to 75% of the plant leaflets; 4 = Very severe chlorosis, complete wilting, or death of the plant in 75% to 100% of the plant leaflets. (Poli et al. 2012) The data were analysed statistically using Analysis of Variance (ANOVA) and Duncan's test (with $P \leq 0.05$ as the significance level).

5. Molecular Characterisation:

5.1. Fungal Mycelia:

The FOL mycelium of the pure culture resulting from the growth of a hyphal tip on potato dextrose broth (PDB) medium (200g potato extract and 20g dextrose per litre) in 100 ml Erlenmeyer flasks at 23°C for 10 days. Each FOL isolate was harvested and deep frozen at -20°C for later genomic DNA extraction.

5.2. Fungal DNA Isolation:

The genomic DNA was isolated according to Khlode *et al.,*2016a. Harvested mycelia were processed by grinding with Cetyl trimethylammonium bromide (CTAB) buffer containing 1.5 M NaCl, 2% (w/v) CTAB, 1% (w/v) SDS, 20 mM EDTA and 100 mM Tris

HCL pH 7.8) supplemented with 0.2% beta-mercaptoethanol, added just before using the sterilised mortar and pestle. Ground mycelium was transferred to a clean 1.5 cm^3 Eppendorf tube. Tubes were vortex for 1-3 sec. and then incubated for 30-60 min. in a water bath at 65 ^oC with continual gentle shaking. Sample tubes were allowed to cool to room temperature for 5 min before an equal volume of phenol: clod chloroform: isoamyl alcohol (25:24:1) mixture was added, and the whole was gently shacked for ten min. The contents of the tube were then centrifuged for 10 min at 11469.6xg. After centrifugation, the upper aqueous layer was transferred to a clean 1.5 cm^3 Eppendorf tube and an equal volume of cooled isopropanol was added to precipitate DNA. The solution was gently mixed by inversing the tube until thread-like strands of DNA formed a visible mass and kept at -20^oC for 30min. The DNA was collected by centrifugation at 31860xg for 15min. The supernatant was decanted and 500µl of 70% ethanol was added to the DNA pellet at room temperature and the tube was gently inverted several times to wash the DNA. The DNA was collected again by centrifugation for 2min at 11469.6 xg. The ethanol supernatant was carefully aspirated using a micropipette. At this point, concern was taken during aspiration to avoid sucking the pellet into the micropipette. The clean DNA pellet was air dried for 15 min after which the DNA pellet was re-suspended into 50µl of TE buffer and stored at -20^oC until use. The DNA concentration was quantified by measuring the absorption of the samples at 260nm using a NanoDrop UV spectrophotometer (Maestrogen, Taiwan).

5.3. Molecular Identification Using Universal Primers:

PCR amplification of ITS rDNA region was carried out using a pair of universal primers, ITS1 and ITS4 (Table 1) following the procedure outlined by White *et al.* (1990). PCR was performed in a total volume of 25µl of PCR reaction consisting of 2µl genomic DNA (50ng), 0.5 µl of each primer "Bio-search Technologies" (10 pmol), 12.5 µl Red PCR master mix (Bio-line) and 9.5µl ddH_2O.

PCR Amplification was performed in a (Techne-Progene) thermocycler. The machine was programmed to follow the following steps: Initial denaturation at 94°C for 4 min, followed by 35 cycles each was made up of three steps, denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10min. The PCR product was separated by electrophoresis on a 1.5 % agarose gel and stained with Red Safe dye in 0.5X TBE buffer and visualised under UV light. A1000 bp DNA marker ladder (Bio-line) was used as a molecular length standard.

The amplified fragment of ITS1-5.8S-ITS2 region (≈550bp) of the test isolate was sent for sequencing to Macrogen, Korea. Were made using Molecular Evolutionary Genetics Analysis Version 10.2.6 (MEGA X) software, and then the phylogenetic tree was constructed using the neighbor-joining method from CLUSTALW alignment (Tamura *et al.*, 2013). The sequence of interest was compared with ten accessions (KF914482.1, MK292327.1, KM983649.1, MK292326.1, DQ452454.1, KU097294.1, KF914475.1, KC478622.1, GU205445.1 and KT153614.1) obtained from GenBank and then deposited in the National Centre for Biotechnology Information (NCBI).

Table Error! No text of specified style in document.**.** ITS amplification primers sequence**.**

Primer name	Oligonucleotide sequence, 5' to 3'	Expected Amplicon size	Reference
ITS1	TCC GTA GGT GAA CCT GCG G	550bp.	White *et al.* (1990)
ITS4	TCC TCC GCT TAT TGA TAT GC		

RESULTS AND DISCUSSION

1. Collection of Diseased Specimens and Fungal Isolation:

Typical symptoms of Fusarium wilt disease were observed in tomatoes. plants growing in five different governorates (Alexandria, EL-Beheira, Kafr El-Sheikh Giza and Assiut) in Egypt during the year, 2021 (Table 2). The symptoms observed on the spotted host plants were recorded by photography (Fig. 1) and parts from the affected areas and soil rhizosphere was cultured for pathogen isolation. The wilt and the associated symptoms c. There was no significant difference, if any, in the observed symptoms expressed by plants collected from different geographical locations.

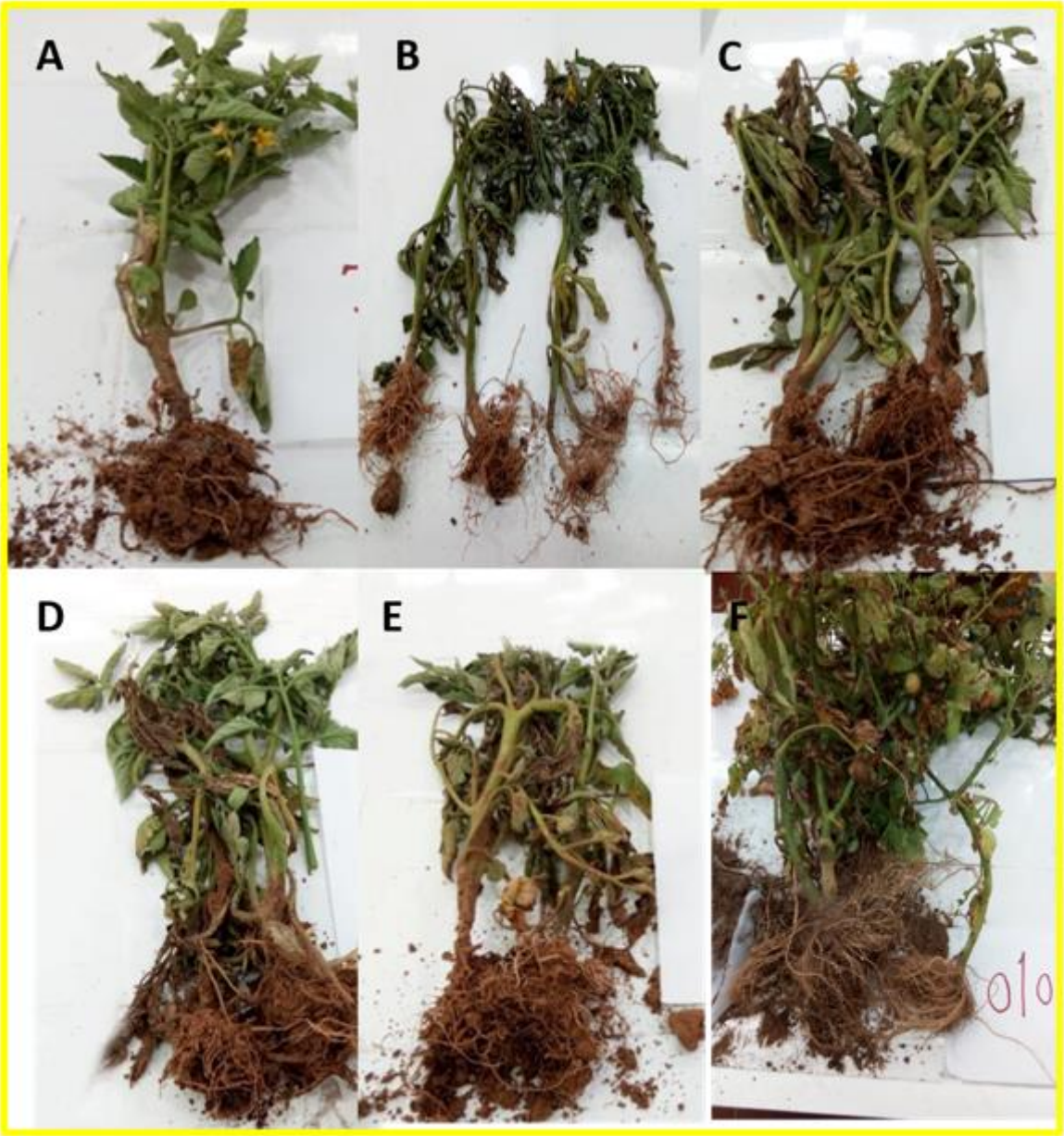

Fig. 1. Symptoms of fusarium wilt disease were observed on representative specimens of naturally infected tomato (*Solanum lycopersicum* L.) plants from various locations in Egypt. A, a specimen from Alexandria; B and C, two specimens from EL-Beheira; D, a specimen from Kafr El-Sheikh; E, a specimen from Giza; and F, a from Assiut."

2. Fungal Isolation:

Eighteen isolates of *Fusarium oxysporum* f.sp. *lycopersici* (FOL) were obtained from different parts, including roots, stems, and rhizosphere soil of naturally infected tomato plants growing in a total of five governorates, namely, Alexandria, EL-Beheira, Kafr Sheikh, Giza, and Assiut (Table 2). The 18 isolates were distributed as 3 from Alexandria, 6 from EL-Beheira, 3 from Kafr El-Sheikh, 2 from Giza and 4 from Assiut. Four isolates were recovered from the rhizosphere while the remaining 14 were isolated from plant specimens.

Table 2. Eighteen isolates of *Fusarium oxysporum* f.sp. *lycopersici* (FOL) obtained from naturally infected tomato plants collected from different governorates of Egypt during the year 2021.

No.	Isolate code.	Source (Host/Habitat)	Geographic origin
1	FOL101	Root	Alexandria
2	FOL102	Root	
3	FOL113	Rhizosphere soil	
4	FOL204	Root	EL-Beheira
5	FOL205	Root	
6	FOL226	Stem	
7	FOL207	Root	
8	FOL208	Root	
9	FOL219	Rhizosphere soil	
10	FOL3010	Root	Kafr El-Sheikh
11	FOL3011	Root	
12	FOL3112	Rhizosphere soil	
13	FOL4013	Root	Giza
14	FOL4014	Root	
15	FOL5015	Root	Assiut
16	FOL5016	Root	
17	FOL5117	Rhizosphere soil	
18	FOL5018	Root	

3. Morphological Identification:

The eighteen isolates of the causal pathogen (FOL) obtained in this study were microscopically examined for identification purposes.

All isolates exhibited morphological features typical to those of *Fusarium oxysporum*. The mycelia were delicate, white to red, and sometimes had a purple tint. They ranged from sparse to abundant in quantity. The fungus produced three types of spores: macroconidia, microconidia and chlamydospores. Macroconidia, which were usually three- to five-septate, fusoid-subulate, and pointed at both ends, were borne on branched conidiophores or on the surface of sporodochia. They had a thin wall and a pedicellate base (Fig. 2A). Microconidia were oval-ellipsoid, straight to curved, and were borne on simple phialides arising laterally. They were usually nonseptate or single septate and occurred in abundance (Fig. 2A). Chlamydospores were abundant, both smooth and rough-walled, and formed terminally or on an intercalary basis (Fig. 2B). They were generally solitary, but occasionally formed in pairs or chains. The *Fusarium oxysporum* isolates displayed a high level of diversity in terms of cultural characteristics and fungal morphology.

Data obtained (Table 3 and Fig.3) indicated that the eighteen FOL isolates vary significantly in growth texture and were grouped into two categories, white dense and white compact. They also varied significantly ($P \leq 0.05$) in mycelial radial growth rate which ranged from 0.6 to 0.9 cm/24h. The conidiospores per Petri plate in a total volume of 10 cm^3 varied

from 1.1 to 2 x 10^{6}. Based on the degree of pigmentation of the mycelium, the isolates were grouped into four categories, light pink, creamy, purple and Red.

Based on the morphological cultural characteristics and microscopic examination (Ali, 2012), the eighteen isolates were identified as *F. oxysporum*.

The highest sporulation rate of $2x10^{6}/cm^{3}$ was detected in two isolates (FOL205 and FOL219) from EL-Beheira. On the other hand, the lowest rate of sporulation was $1.1x10^{6}/cm^{3}$ in four isolates (FOL102, FOL207 and FOL5117).

Table 3. Morphological features and radial growth rates of eighteen isolates. of *Fusarium oxysporum* f. sp. *Lycopersici* obtained from naturally infected tomato plants grown in five different governorates of Egypt during the year 2021.

Isolate code.	Texture	Growth rate/day	Pigments	Conidiospores /10ml (x 10^{6})
FOL101	White dense	0.6	light pink	1.2
FOL102	White compact	0.7	creamy	1.1
FOL113	White dense	0.6	purple	1.4
FOL204	White dense	0.6	light pink	1.4
FOL205	White compact	0.9	Red	2
FOL226	White compact	0.7	light pink	1.7
FOL207	White compact	0.7	light pink	1.1
FOL208	White compact	0.6	purple	1.9
FOL219	White compact	0.8	Red	2
FOL3010	White dense	0.6	purple	1.4
FOL3011	White dense	0.7	creamy	1.8
FOL3112	White dense	0.7	creamy	1.9
FOL4013	White dense	0.8	light pink	1.3
FOL4014	White compact	0.6	light pink	1.2
FOL5015	White compact	0.7	purple	1.9
FOL5016	White compact	0.7	light pink	1.7
FOL5117	White dense	0.6	light pink	1.1
FOL5018	White dense	0.6	purple	1.6

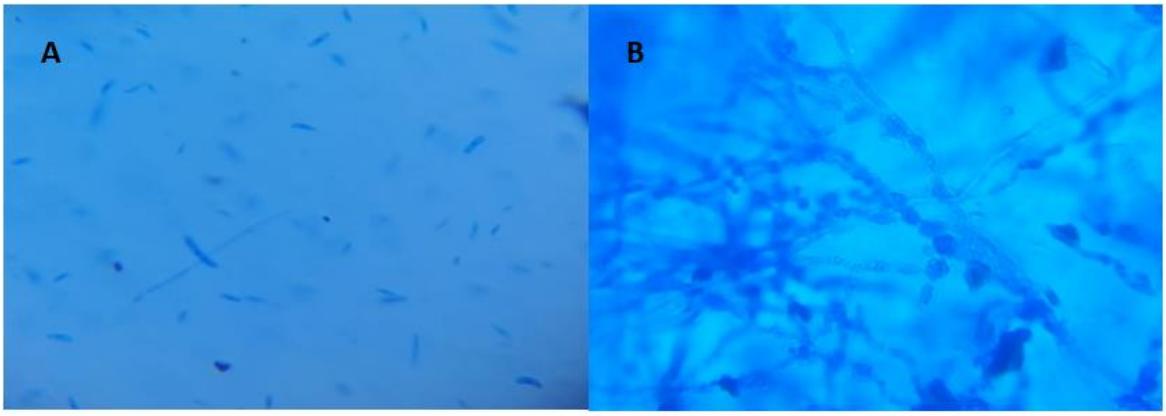

Fig. 2: A representative isolate of *Fusarium oxysporum* f. sp. *Lycopersici* out of eighteen isolates obtained from naturally infected tomato plants grown in five different governorates of Egypt during the year 2021 as seen by light microscopy (400X). A, Macroconidia (fusiform shape, multicellular) and microconidia (unicellular) and B, chlamydospores stained with lactophenol-cotton blue.

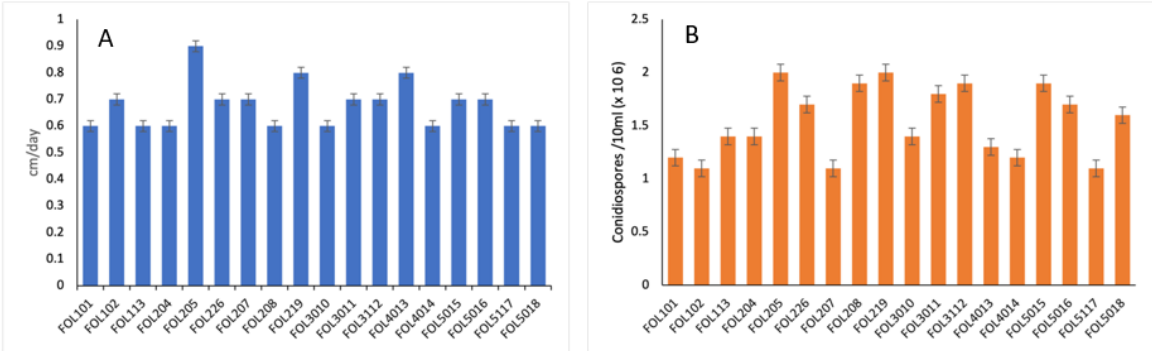

Fig. 3. A, Radial growth rate (cm/ day) of eighteen isolates of *Fusarium oxysporum* f. sp. *Lycopersici*. B, Concentration of conidiospores in 10 cm^3 (x 10^6).

4. Pathological Characterization:

The initial indications of wilt disease typically appeared 15 to 20 days after the plants were artificially inoculated. Virulence testing on tomato plants showed variations in symptomology on the aerial parts of the plants following artificial inoculation with the FOL. At the early stage of infection, the lower leaves displayed yellowing and in severely affected plants they dried out, while in the later stages, the whole plant lost its integrity and leaves manifested typical wilting. The results of the pathogenicity tests of the 18 FOL isolates carried out on four different tomato cultivars are given in Table 4. Based on the mean disease severity (MDS), the eighteen FOL isolates displayed varying levels of virulence on the test tomato varieties, which were categorised as low (MDS: < 25%), moderate (MDS: 25–50%), or high (MDS: > 50%). The isolates were then classified into three groups: highly virulent (8 isolates), moderately virulent (4 isolates), and weakly virulent (6 isolates). No symptoms were observed on the control uninoculated tomato seedlings. The Root-dip inoculation test revealed that all isolates were *Fusarium oxysporum* f. sp. *lycopersici*. All isolates were successfully reisolated from the artificially inoculated plants expressing the characteristic symptoms of *Fusarium*-wilt disease, thereby fulfilling Koch's postulates.

Table 4. Virulence assessment of eighteen isolates of *Fusarium oxysporum* f. sp. *lycopersici*.

Isolate code.	Geographic Location (Governorate)	Disease severity observed on tomato cultivars (Disease index 0-100%)				Mean	Virulence grade
		030	86	Dussehra	111		
FOL101	Alexandria	63.25	68.01	67.97	75.69	68.73^{b}	High
FOL102		21.16	32.56	25.56	43.6	30.72^{f}	Moderate
FOL113		18.2	29.29	25.82	37.21	27.63^{f}	Moderate
FOL204	EL-Beheira	39.32	52.5	48.46	68.89	52.29^{e}	High
FOL205		77.37	83.92	82.64	83.89	81.95^{a}	High
FOL226		8.17	14.47	10.41	10.51	10.89^{g}	Low
FOL207		52.96	47.2	48.54	46.34	48.76^{cd}	Moderate
FOL208		9.44	14.16	14.96	18.00	14.14^{g}	Low
FOL219		65.85	71.97	63.92	66.57	67.07^{b}	High
FOL3010	Kafr El-Sheikh	0	3.03	2.9	3.96	2.4725^{h}	Low
FOL3011		9.15	13.25	11.71	15.3	12.35^{g}	Low
FOL3112		16.8	31.14	24.89	25.00	24.45^{f}	Low
FOL4013	Giza	53.79	49.22	46.46	52.13	50.4^{cd}	High
FOL4014		55.79	53.94	53.14	59.93	55.7^{c}	High
FOL5015	Assiut	36.3	47.79	41.98	45.62	42.92^{e}	Moderate
FOL5016		18.77	22.22	15.28	24.77	20.26^{f}	Low
FOL5117		47.73	49.93	63.57	55.59	54.205^{d}	High
FOL5018		56.79	67.46	53.62	66.09	60.99^{c}	High

The disease index for the isolates listed was assessed on a 0 to 100% scale where 0, refers to healthy plants; 12.5, to plants growing regularly; 25, to plants with slight leaf chlorosis and reduced growth; 50%, to plants showing chlorosis and 50% growth reduction, as compared to the healthy control, and initial symptoms of wilting; 75, plants with severe leaf chlorosis, growth reduction and wilt symptoms; 100, plants totally wilted and dead. Analysis of variance (ANOVA) was performed and within each column, mean values followed by a common letter do not differ statistically according to Duncan's multiple range test (DMRT) ($P \leq 0.05$).

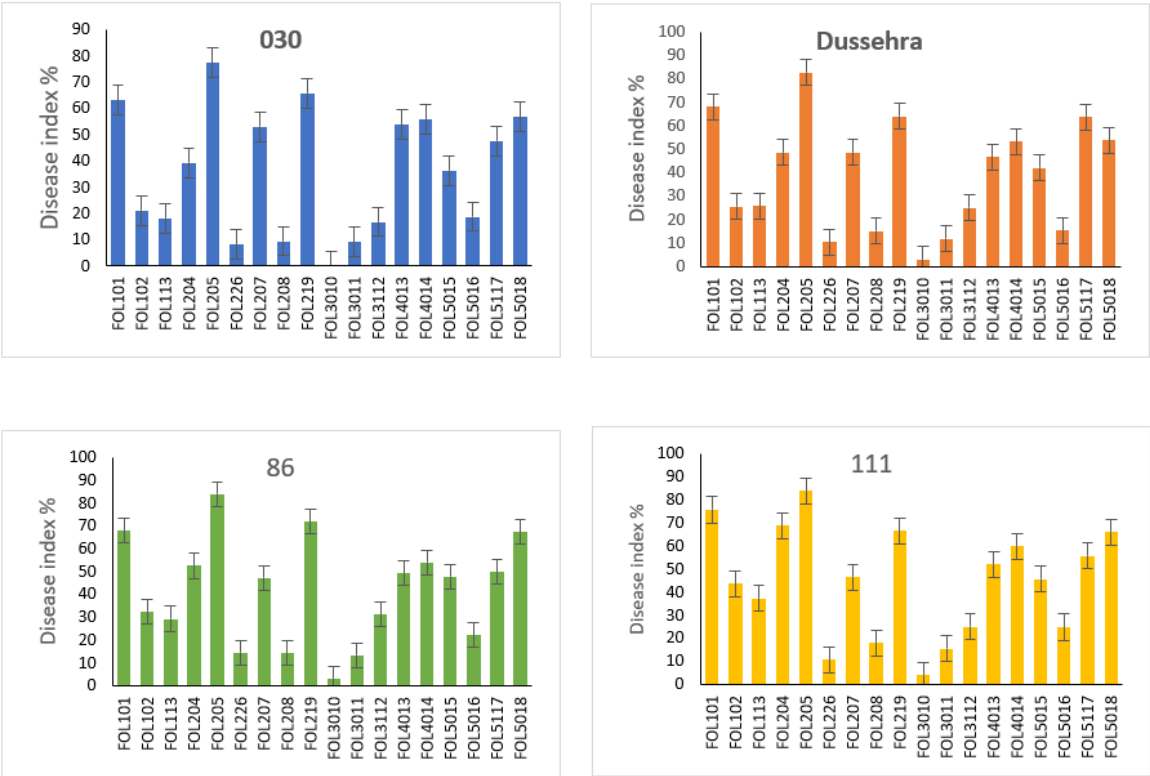

Fig. 4: A bar chart showing the percentage of wilt disease index on four commercial tomato cultivars (030, Dussehra, 86, and 111).

Previous studies (Amini. 2009; Jacobs, *et al*., 2013; Khlode *et al*., 2016a; Rodríguez-Molina *et al*., 2003; Song *et al*., 2004) have identified *Fusarium oxysporum* f.sp. *lycopersici* as the primary cause of tomato wilt in Egypt and other parts of the world. Pathogenicity tests are commonly used to distinguish between different pathogenic *Fusarium* isolates, but they do not provide information on genetic relatedness among isolates of the same physiologic race or forma specialis. The results obtained in the present study indicated that isolate FOL205, obtained from El-Baheria, was the most aggressive one, while isolate FOL3010, obtained from Kafr El-Sheikh, was the weakest among the eighteen isolates tested. However, the two isolates (FOL4013 and FOL4014) from Giza demonstrated high virulence and the three isolates (FOL3010, FOL3011 and FOL3112) from Kafr El-Sheikh exhibited low virulence. The isolates from El-Behira and Assiut showed intermediate virulence grades across the spectrum.

The relationship between the aggressiveness of virulence and conidiospore formation is clear from the data in Table 3 and Figure 4. The two FOL isolates with the high level of sporulation (2x 106/ml) (Table 3) exhibited a high level of virulence as shown in Figure 4. This suggests that the conidiospore formation rate could likely be a determining factor in determining the degree of virulence since the conidiospore can spread intercellularly through the xylem tissue in the infected plants.

5. Molecular Identification:

5.1. Molecular Identification of FOL Isolates Was Carried Out Using A Pair of Universal Primers Via Polymerase Chain Reaction (PCR): The amplification of ITS1-5.8S-ITS4 regions of five selected isolates (FOL204, FOL207, FOL3010, FOL4014 and FOL5018) was carried out using the primers, ITS1 and ITS4. PCR produced an amplicon with an expected molecular length of approximately 550 bp (Fig. 5).

Several molecular methods can also be used in the identification process as described by (Mostafa *et al.*, 2022) and (Behiry *et al.*, 2023), however amplification of the ITS region has shown consistency with various FOL isolated worldwide.

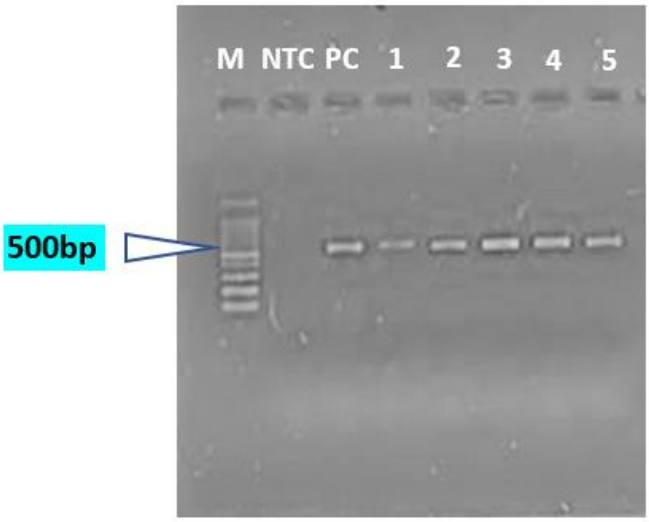

Fig. 5. Agarose (1.5%) gel electrophoresis showing a single PCR amplification product of 550bp long using ITS1-ITS4 pair of primers. Left lane (M), is loaded with a 100 bp DNA ladder marker; NTC, no template control (a reaction mixture lacking DNA template); PC positive control; lanes1 to 5 correspond to the 5 test isolates (FOL204, FOL207, FOL3010, FOL4014 and FOL5018).

5.2. DNA Sequencing and Phylogenetic Analysis of the ITS1 5.8S ITS4 Region:

Sequence analysis of the amplified region 5.8S flanked by the complementary sequence to ITS1 and ITS4 pair of primers revealed that all isolates tested were *Fusarium oxysporum* f. sp. *Lycopersici* (FOL) according to the percentage of identity imported from GenBank using BLAST search. The identity of all isolates tested was 100% with an e-value of zero. Considerable variation was observed when the obtained sequences of the amplified region (ITS1-5.8s-ITS4) were aligned.

A phylogenetic tree (Fig. 6) has been constructed using the five Egyptian FOL isolates used in this study against 9 identified FOL isolates obtained via BLAST search. These 9 isolates were used to generate the tree as they showed the highest similarity with the sequences of the 5 Egyptian FOL isolates. As shown in Fig. 4, the tree obtained is divided into two main branches, the first contains isolate KT153614.1 and the other branch contains the rest of the isolates. The second branch is further divided into 4 sub-clusters with FOL 4014 and FOL5018 being the most relative to each other. Surprisingly, these two isolates are from Giza and Assiut, two geographically distant governorates. Likewise, the two isolates, FOL207 and FOL3010 obtained from El-Beheira and Kafr El-Sheik were found to be close relatives as they shared the same cluster, but the fact that the two governorates are relatively close to each other, this result does not elicit any wonder. Noticeably, isolate FOL204 from El-Beheira appeared to be in a separate cluster from all the other four isolates indicating its less relativeness. It is, thus, likely that this FOL isolate has undergone some changes in its ITS sequence. Interestingly, the sequencing data from these 5 isolates were found to be new as there were no similar sequences reported from Egypt in the NCBI database. These data show the degree of relativeness among some Egyptian FOL isolates. However, more work is still needed as sequencing the rest of the isolates can be included and compared.

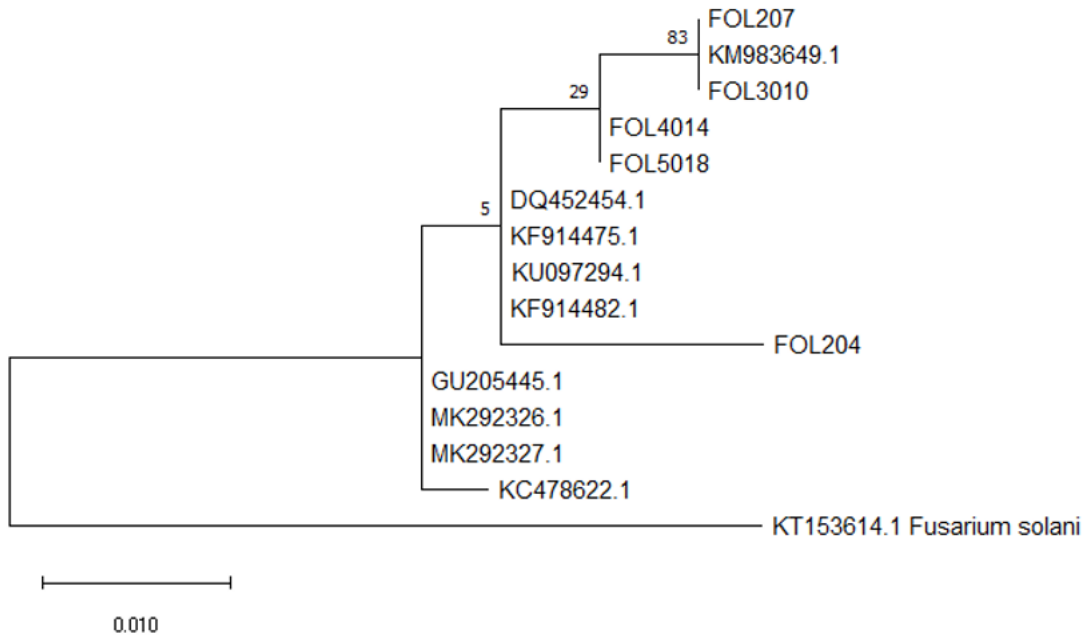

Fig. 6: Phylogenetic tree constructed using MEGA X software programme (neighbour-joining tree method) based on nucleotide sequence alignment of PCR product (≈550 bp) representing the amplified sequence of ITS1-5.8-ITS4 region of rDNA using specific primers (ITS1/ITS4) as compared to other selected isolates from GenBank.

Conclusion:

The current study has proven that *Fusarium oxysporum* f. sp. *Lycopersici* (FOL), the causal agent of fusarium wilt disease, is a ubiquitous pathogen infecting tomato plants in Egypt. Yet, the pathogen isolated from different geographical locations within the country demonstrates a high degree of morphological, pathological and molecular variability. It is believed that the results obtained in this investigation particularly those relevant to genetic analysis could be utilised to develop new strategies for effectively controlling this pathogen and might also be beneficial for plant breeding purposes to develop disease-resistant varieties.

REFERENCES

Ali S. (2012). “Interaction of Polygalacturonase Enzymes from *Fusarium oxysporum* with Tomato Polygalacturonase Inhibiting Protein.” *African Journal of Agricultural Reseearch*, 7(13).

Amini, J. (2009). “Physiological Race of *Fusarium oxysporum* f.sp. *lycopersici* in Kurdistan Province of Iran and Reaction of Some Tomato Cultivars to Race 1 of Pathogen.” *Plant Pathology Journal*, 8(2): 68–73.

Behiry, S., Seham A. Soliman, Magdy A. Massoud, Moawad Abdelbary, Ahmed M. Kordy, Ahmed Abdelkhalek, and Ahmed Heflish. 2023. "Trichoderma pubescens Elicit Induced Systemic Resistance in Tomato Challenged by Rhizoctonia solani" *Journal of Fungi*, 9, no. 2: 167. https://doi.org/10.3390/jof9020167.

Chakraborty, N., Swarnendu C., and Krishnendu A. (2017). “Biochemical Basis of Improvement of Defense in Tomato Plant against Fusarium Wilt by $CaCl_2$.” *Physiology and Molecular Biology of Plants* 23(3):581–96.

El-Mohamedy, R. S. R., Jabnoun-Khiareddine, H. and Daami-Remadi. M. (2014). “Control of Root Rot Diseases of Tomato Plants Caused by *Sclerotium Rolfsii* Using Different Chemical Plant Resistance Inducers.” *Tunisian Journal of Plant*

Protection 9 (1): 45–55.

Erba, D., Cristina C. M., Ribas-Agustí A., Caceres R. C., Marfa O. and Castellari, M. (2013). "Nutritional Value of Tomatoes (Solanum Lycopersicum L.) Grown in Greenhouse by Different Agronomic Techniques." *Journal of Food Composition and Analysis* 31(2): 245–51.

FAO. 2019. The State of Food and Agriculture 2019. Moving forward on food loss and waste reduction

Fakhouri, W. and Buchenauer, H. (2003). "Characteristics of Fluorescent Pseudomonad Isolates towards Controlling of Tomato Wilt Caused by *Fusarium oxysporum* f.sp. *lycopersici*." *Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz* 110(2): 143–56.

Gonzalez-Cendales, Y., Catanzariti, A. M., Baker, B., Mcgrath, D. J. and Jones, D. A (2016). "Identification of I-7 Expands the Repertoire of Genes for Resistance to Fusarium Wilt in Tomato to Three Resistance Gene Classes." *Molecular Plant Pathology,* 17 (3): 448–63.

Hahn, M. G. (1996). "Microbial Elicitors and Their Receptors in Plants." *Annual Review of Phytopathology* 34(30): 387–412.

Hansen, H. N. and **Snyder, W. C. (**1940). "The Species Concept in Fusarium Author (s): W . C . Snyder and H . N . Hansen Published by : Botanical Society of America Stable URL : Http://Www.Jstor.Org/Stable/2436688 ." *American Journal of Botany* 27(2): 64–67.

Jacobs, A., Govender R. and Van Heerden, S. W. (2013). *Fusarium oxysporum* f.sp. *lycopersici* Race 3 Causing Tomato Wilt in South Africa." *Australasian Plant Disease Notes* 8(1): 145–47.

Jaiswal, A. K., Alkan, N., Elad, Y., Sela, N., Philosoph, A. M., Alkan, N., Yigal Elad, Noa Sela, Philosoph, A. M., Graber, E. R. and Frenkel. O. (2020). "Molecular Insights into Biochar-Mediated Plant Growth Promotion and Systemic Resistance in Tomato against Fusarium Crown and Root Rot Disease." *Scientific Reports* 10(1): 1–15.

Khlode, R. S. A., Abdel-Gawad, T. I., El-Bana, A. A. and Galal, A. A. (2016a). Fusarium oxysporum f.sp. lycopersici (Fol) Race 1 and 3 As Wilt-Incitants To Tomato Plants Growing At El-Minia Governorate, Egypt." Minia Journal of Agriculture Research & Development, Vol. (36),(2):229–44.

Khlode, R. S. A., Abdel-Gawad, T. I., El-Bana, and Galal, A. A. (2016b). Fusarium oxysporum f.sp. lycopersici (fol) race 1 and 3 as wilt-incitants to tomato plants growing at el-minia governorate, egypt." *Minia Journal of Agriculture Research & Development,* Vol. 36,(2): 229–44.

Manikandan, R., Harish, S., Karthikeyan, G. and Raguchander, T. (2018). "Comparative Proteomic Analysis of Different Isolates of *Fusarium oxysporum* f.sp. *lycopersici* to Exploit the Differentially Expressed Proteins Responsible for Virulence on Tomato Plants." *Frontiers in Microbiology* , 9: p.420.

McGovern, R. J. (2015). "Management of Tomato Diseases Caused by *Fusarium oxysporum*". *Crop Protection* 73: 78–92.

Mostafa, Y. S., Alamri, S. A., Alrumman, S. A., Hashem, M., Taher, M. A., and Baka, Z. A. (2022). "In Vitro and In Vivo Biocontrol of Tomato Fusarium Wilt by Extracts from Brown, Red, and Green Macroalgae." *Agriculture (Switzerland)* 12(3): 1–18.

O'Donnell, K., Kistler, H. C., Tacke, B. K. and Casper, H. H. (2000). "Gene Genealogies Reveal Global Phylogeographic Structure and Reproductive Isolation among Lineages of Fusarium Graminearum, the Fungus Causing Wheat Scab." *Proceedings of the National Academy of Sciences of the United States of America* 97(14): 7905–10.

Poli, A., Gilardi, G., Spadaro, D., Gullino, M. L. and Garibaldi, A. (2012). "Molecular Characterization of *Fusarium oxysporum* f.sp.*Cichorii* Pathogenic on Chicory (Cichorium Intybus)." *Phytoparasitica* 40(4): 383–91.

Quinet, M., Angosto, T., Yuste-Lisbona, F. J., Blanchard-Gros, R., Bigot, S., Martinez, J. P. and Lutts, S. (2019). "Tomato Fruit Development and Metabolism." *Frontiers in Plant Science* 10 (November):1–23.

Ramamoorthy, V., Raguchander, T., and Samiyappan, R. (2002). "Induction of Defense-Related Proteins in Tomato Roots Treated with Pseudomonas Fluorescens Pf1 and *Fusarium oxysporum* f.sp. *lycopersici*." *Plant and Soil* 239(1): 55–68.

Rodríguez-Molina, M. C., Medina, I., Torres-Vila, L. M., and Cuartero, J. (2003). "Vascular Colonization Patterns in Susceptible and Resistant Tomato Cultivars Inoculated with *Fusarium oxysporum* f.sp. *lycopersici* Races 0 and 1." *Plant Pathology* 52(2): 199–203.

Singha, I. M., Kakoty, Y., Unni, B. G., Das, J. and Kalita, M. C. (2016). "Identification and Characterization of Fusarium Sp. Using ITS and RAPD Causing Fusarium Wilt of Tomato Isolated from Assam, North East India." *Journal of Genetic Engineering and Biotechnology* 14(1): 99–105.

Song, W., Zhou, L., Yang, C., Cao, X., Zhang, L. and Liu, X. (2004). "Tomato Fusarium Wilt and Its Chemical Control Strategies in a Hydroponic System." *Crop Protection* 23(3): 243–47.

Srinivas, C., Devi, D. N., Murthy, K. N., Mohan, C. D., Lakshmeesha, T. R., Singh, B. P., Kalagatur, N. K., Niranjana, S. R., Hashem, A., Alqarawi, A. A., Abd Allah, T. E. F. and Nayaka, S. C. (2019). "*Fusarium oxysporum* f.sp. *lycopersici* Causal Agent of Vascular Wilt Disease of Tomato: Biology to Diversity– A Review." *Saudi Journal of Biological Sciences* 26(7): 1315–24.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). "MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0." *Molecular Biology and Evolution* 30(12): 2725–29.

White, T.J. (1990) Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: PCR Protocols, a Guide to Methods and Applications, 315-322.