

ORIGINAL PAPER

Biodiversity of *Fusarium oxysporum* Isolated from Diseased Chickpea and Detection of Resistance Sources to Some Egyptian Chickpea Cultivars

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ABSTRACT

Fusarium wilt caused by *Fusarium oxysporum* (*Fo*) is the maximum critical soil borne diseases of chickpea in Egypt and many other countries in the world. Sixteen *F. oxysporum* (*Fo*) isolates were collected from 9 Governorates in Egypt isolated from wilted chickpea plants. Identification of these isolates was achieved by variation in morphological characters *i.e.*, growth rate, growth habits, pigments, sporulation, and the morphological identifications were further confirmed by molecular method using specific ITS primers. Virulence of these isolates was classified as high virulence (*Fo*7, *Fo*8 and *Fo*10), moderate (*Fo*2, *Fo*3, *Fo*6, *Fo*9, *Fo*11, *Fo*13, *Fo*14, *Fo*15 and *Fo*16) and low (*Fo*1, *Fo*4, *Fo*5, *Fo*12). Host response of chickpea cultivars infected with *Fo*7 showed a moderate resistance (Giza-2 and Giza-531) and susceptible ones (Giza-195, Giza-1 and Giza-88). Mechanism underline induce disease resistance revealed that resistant chickpea cultivars contained high activities peroxidase, polyphenol oxidase, catalase, Phenylalanine ammonia lyase and Phenolic compounds than susceptible cultivars.

Keywords: Chickpea, *Cicer arietinum*, *Fusarium oxysporum*, Biodiversity, Oxidative enzyme, Total phenolic.

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) one of the most important legume crops in Egypt as it offers human nutrition with vegetable protein. Chickpea is a good source of protein, carbohydrate, minerals (phosphorus, calcium, magnesium, iron and zinc) and -carotene moreover its protein quality is better than most other legumes (Jukanti, *et al.*, 2012). It is also considered as an important source of human food and animal feed that at the same time helps in managing the soil fertility particularly in dry lands. Chickpea can be a very useful legume crop for incorporation into short-term rotation and for fixation of nitrogen in soil and its fertility (Ali and Kumar, 2009).

The crop is known to be affected by number of pathogens *i.e.*, fungi, bacteria, viruses, and

nematodes. The major constraint to chickpea production is its susceptibility to soil borne diseases (Pande *et al.*, 2005). Soil borne diseases such as wilt, dry root rot, black root rot, collar rot, and stem rot are the major cause in reducing the yield of the crop, its production is badly reduced by *Fusarium* wilt disease caused by *F. oxysporum* and is considered as one of the most important biotic stresses of chickpea. The average annual yield losses due to wilt have been estimated to 10 to 90% and sometimes escalate to 100% when the relative humidity is greater than 60% and temperature ranges between 10 and 25°C (Badawi *et al.*, 2007).

Fusarium oxysporum is seed borne as well as soil borne in nature. Due to the dynamic nature of this soil borne pathogen, the wilt disease manifests as mortality of young seedlings (within 25 to 30 days) after sowing to wilting or death of adult plants. Early wilting causes more loss than late wilting. Nevertheless, the seeds from late-wilted plants are lighter, rough and dull than those from healthy plants (Haware and Nene., 1980). Wilting may initially affect only one side of the plant. The fungus infects chickpeas via the roots and moves throughout the host's vascular system. Cell wall degrading enzymes produced by the pathogen break down the host cell walls to form gels that block the plant's transport systems and cause yellowing and wilting symptoms. Affected seedlings can be identified approximately 3 weeks after sowing and show symptoms such as drooping

and paler coloured leaves. There are no superficial signs of rotting, but when roots are split longitudinally there is a brown to black discolouration of internal tissue (Nene and Reddy, 1987).

The fungus can be transmitted by seed and may survive in plant debris in soil. It was demonstrated that the fungus chlamydospores were found free in soil (Haware *et al.*, 1996), in the hilum of the seed (Haware *et al.*, 1978), in cotyledons and axis (Shakir and Mirza, 1994). The primary infection is mainly through chlamydospores or mycelia. The conidia of the fungus are short lived; however, the chlamydospores can remain viable up to the next crop season. The pathogen can survive as mycelium and chlamydospores in seed and soil and also on infected crop residues, roots and stem tissue buried in the soil for more than 6 years, even in the absence of the host (Castro *et al.*, 2008 and Salem *et al.*, 1991), therefore it is impracticable to control the disease by using fungicides and through crop rotation.

To control the spread of this fungus, the prime requirement is to identify this fungus up to the species level (Abd-Hady *et al.*, 2000). Morphological and microscopically characterization is among the main methods employed in the identification of phytopathogenic fungi. It is used widely, and various parameters such as colonies pigmentation, texture, shape, marginal form and mycelial growth rate, process of formation, spore size and shape are involved (Burgess *et al.*, 1995). But due to their great similarity, the differentiation of fungi through morphological characters is imprecise and confusing.

For the better and more precise result, an advanced DNA-based analysis provides more accurate rapid identification and characterization of the species. Phylogenetic analysis based on DNA sequence data of internal transcribed spacer (ITS) regions has become a diagnostic routine for the detection, identification, classification and phylogenetic analysis of many fungi at the species level data (Hafizi *et al.*, 2013 and Zhao *et al.*, 2014) and has made significant contributions to the better understanding of the systematics of *Fusarium* spp. Molecular data in combination with distinctive morphological characters usually have been provided more clear way to distinguish new species of *Fusarium*.

Once the genetic proximity or similarity relationships of pathogens and DNA sequence-based isolates were determined, the use of

resistant cultivars can be employed as one of the cheapest, economical and the most ideal way of managing chickpea wilt (Salem *et al.*, 1991 and Khattab and Omar., 1992). Resistant cultivars are the most practicable, feasible, and economical approach for the management of wilt disease chickpea, but only a few sources with low level of genetic resistance are available, so there is a need to identify the resistant sources in chickpea.

Biochemical analysis is important to understand the resistant mechanism before and after infection of plant and to isolate the resistance sources (Jyothi *et al.*, 2018 and Narula *et al.*, 2020). It is considered as a direct control tactic in integrated plant disease management that involve the use of additive or synergistic combinations of biotic, cultural, and chemical control measures (Jiménez *et al.*, 2015 and Landa *et al.*, 2004). Possibly, resistant cultivars as control strategy will become increasingly widespread in the coming years in order to satisfy consumers' demand for healthier foods and better environmental quality through new forms of agricultural production, *viz.*, sustainable agriculture, organic agriculture and ecological agriculture (Abd-Hady *et al.*, 2000).

This study will reveal how much diversity exists in the isolates of *F. oxysporum*. Biochemical and molecular genetic analysis will be done simultaneously to understand the resistant mechanism before and after infection of plant, identify the resistant sources in chickpea.

MATERIALS AND METHODS

Isolation, purification, and identification of the associated fungi:

A total of 16 isolates of fungi were isolated from various sources of diseased chickpea plants from wide range of Geographic Location and Governorates in Egypt.

Roots and basal stems of diseased chickpea plants showing typical symptoms of wilt disease were washed carefully with running tap water. Plants in the diseased samples were cut into small pieces, surface sterilized by immersing in 2% sodium hypochlorite for 3 min, and washed twice in sterilized distilled water, then dried between two sterilized filter paper and plated on PDA medium, then incubated at 25±2°C for 7 days. Emerged fungi were isolated and purified on PDA plates; all cultures were derived from single spore and then identified according to Nelson *et al.* (1983) and Booth (1971). Identification of pathogen was confirmed in

Mycological Res. and Disease Survey Department, Plant Pathology Res. Institute, Agric. Res. Center (ARC), Giza, Egypt. The different *Fusarium* isolates were coded with F0-1 to 16, maintained on PDA slants and kept at 4°C for further studies.

Cultural and morphological variability:

In order to study cultural characteristics, 5 mm mycelia bits of each isolate was taken from the actively growing cultures and centrally placed on 90 mm Petri plates containing sterilized PDA medium. After inoculation Petri plates were incubated at 26±1°C for 7 days. Each plate is replicated three times. After seven days radial growth of the fungal isolates was recorded. Other characteristics *viz*: pigmentation, sporulation of different isolates was recorded by observing culture plate after complete growth of the mycelium which showed slight pinches of color.

The morphological studies were carried out by cutting a small piece of mycelium after fourteen days old pure culture plates using a sterile needle and transferred onto a cleaned glass slide. The samples were taken from five different positions of the culture plate, four from adjacent side and one from middle. The mycelial growth was stained with 0.1 % lactophenol cotton blue and observed under compound microscope.

Molecular identification:

Genomic DNA were extracted from fungal samples using a DNeasy Blood & Tissue extraction kit (Qiagen, CA, USA) according to the manufacturer's instructions. In order to identify the samples by molecular methods, PCR amplification and sequencing of ITS rRNA were carried out.

The primers used for amplification of the ITS rRNA gene using ITS1 forward (5'-TCCGTAGGTGAACCTGCGG-3'). (White *et al.* 1990) and ITS4-B reverse (5' CAGGAGACTTGTACACGGTCCAG-3') (Gardes and Bruns, 1993).

DNA amplification was performed in a final volume of 50µl containing 2µl (30ng) of DNA template, 25µl of AmpliTaq Gold® 360 Master Mix (Applied Biosystems), 1µl (10 pmol/ µl) of each forward and reverse primer in addition to 21µl of nuclease free water. Amplification was carried out according to (Kamhawy *et al.*, 2011). The PCR products were visualized on 1.5% agarose gel using SYBR Green staining. They were purified using ExoSAP-IT and then were sequenced by a Sanger's Dideoxy method (Sanger *et al.*, 1974). (The Big Dye Terminator

v3.1 cycle sequencing kit (Applied Biosystems) and an automated DNA sequencer (Applied Biosystems 3130 Genetic Analyzer) were used for sequencing. Sequences obtained were analyzed together with sequences available in GenBank (NCBI) using BLAST (Basic Local Alignment Search Tool) program.

Pathogenicity tests for *F. oxysporum* isolates:

The sixteen isolates of *F. oxysporum* representing the surveyed nine Egyptian governorates were chosen to determine their pathological ability on chickpea cultivar Giza-1 (obtained from Field Crops Res. Inst., ARC., Giza, Egypt) by using the following technique:

Inocula of sixteen isolates were prepared from 21-day-old cultures grown in liquid V8 juice medium at 25±2°C at 100 rpm under continuous cool fluorescent light. The mycelia of the cultures were removed by passing through four layers of cheesecloth, and the concentration of spores present in the liquid medium was adjusted to 1×10⁶ spore per ml using a hemacytometer (Sharma *et al.*, 2005).

The tested cultivar Giza-1 was planted in autoclaved soil at 121°C for 60 min for three continuous days in 30 cm sterilized plastic pots with 10 seeds/pot of chickpea cultivar (Giza-1). Pots were sterilized by immersion in 5% formalin solution, ten days before use. Seeds were surface sterilized by immersion in 2% solution of sodium hypochlorite for 3 minutes and washed in distilled water before planting. The pots were kept under greenhouse conditions for 15 days. Twenty days after sowing, potted soil was infested with spore suspension of *F. oxysporum* at concentration 1×10⁶ spore/ml (50 ml/pot). Three pots were used for each treatment as replicates. Data were recorded after 25 days from inoculation for early wilt and 60 days after inoculation for late wilt Allen and Lenné (1998).

Disease Severity: 0-4 scale (0 = 0%; 1 = 1 - 33%; 2 = 34 - 66%; 3 = 67 - 100%; 4 = Dead plant) based on acropetally yellowing and necrosis was used. In the sensitive chickpea cultivar, the isolates having the value of 3.1-4.0 are considered as highly virulent (HV), the isolates with 1.1-3.0 scale values are moderately virulent (MV) and the isolates with 0.0-1.0 value are low virulent (LV) or non-pathogen (Trapero-Casas and Jimenez-Diaz, 1985).

Chickpea cultivars reaction to the infection by *F. oxysporum*:

Five chickpea cultivars, Giza-1, Giza-2, Giza-88 Giza-195, Giza 531 obtained from Field Crops Res. Inst., ARC., Giza, Egypt) were evaluated under greenhouse conditions, for the

reaction against the highly pathogenic *Fusarium* isolate F07. Procedures of inoculum preparation, soil infestation, seed sowing and experiment setup were followed as mentioned before and scored according to Haware and Nene (1982) as follows:

R = Resistant (0-20 % infected plants)

M = Moderately susceptible (21-50 % infected plants)

S = Susceptible (>50% infected plants)

Biochemical analysis:

These experiments were carried out at the Central Lab of Plant Pathology Res. Institute. ARC.

Sample collection:

Samples of five chickpea cultivars seedlings (shoot) were collected after 2, 5, and 8 days of inoculation with the pathogen. Also, healthy seedling was used for extraction.

Extraction and assays of certain enzymes:

Extraction and assay of peroxidase:

Shoot samples (1 g) were homogenized in 2 ml 0.1 M phosphate buffer, pH 7.0 at 4°C. The homogenate was centrifuged at 14000 g at 4°C for 15 min and the supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1% H₂O₂. The reaction mixture was incubated at 28±2°C for 5 min and reaction was stopped by adding 1mL of 1M HCl (Yingsanga *et al.*, 2008). Changes in A₄₂₀ due to pyrogallol oxidation were recorded. The enzyme activity was expressed as changes in A min⁻¹ g⁻¹ f. wt (Hammerschmidt *et al.*, 1982).

Extraction of polyphenol oxidase (PPO) and catalase (CAT):

Extraction was done following the Mayer *et al.* (1965), method. Shoot samples (1 g) were homogenized in 2 ml 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 15000 g for 15 min at 4 °C. The supernatant was used as the enzyme source of polyphenol oxidase and catalase.

Activity of PPO:

It was determined following the method according by Mayer *et al.*, (1965). The reaction mixture consisted of 200 µl of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 200 µl of 0.01 M catechol were added and the reaction was terminated after 10 min by adding 1 ml of 1M HCl (Yingsanga, *et al.*, 2008). Change in A₄₉₅ was recorded. The enzyme activity was expressed as change in oxidation of catechol min⁻¹ g⁻¹ f. wt.

Catalase assay:

Catalase activity was assayed following the method according by Ueda *et al.* (1990). In a quartz cuvette (10-mm light path) we added 680 µl of 50 mM potassium phosphate buffer (pH 7.2) and 480 µl of 40 mM hydrogen peroxide. The mixture was then incubated for 2.5 min at 30°C. After incubation period, the reaction was initiated by the addition of 200 µl enzyme extract and then the reaction was stopped by vigorous boiling for 10 min (Garge, *et al.*, 1999). The decrease in absorbance at 240 nm was followed spectrophotometrically. Activity of catalase was represented as change in absorbance at 240 nm min⁻¹ g⁻¹f.wt.

Extraction and assaying the activity level of phenylalanine ammonia lyase:

Shoot samples (1g) were homogenized in 3 ml of ice cold 0.1 M sodium borate buffer, pH 7.0, containing 1.4mM 2-mercaptoethanol and 0.1g insoluble polyvinylpyrrolidone. The extract was filtered through cheese cloth and the filtrate was centrifuged at 16000 g at 4°C for 15 min. The supernatant was used as the enzyme source (Dickerson *et al.*, 1984). Activity of PAL was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid as described by (Dickerson *et al.*, 1984). A sample containing 0.5 ml of enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8, and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 60 min at 30°C. The reaction was terminated by the addition of 35% (w/v) trifluoroacetic acid (Rivero *et al.*, 2001). The optical density value was recorded at 290 nm. Enzyme activity was expressed as change in readings min⁻¹ g⁻¹ f. wt.

Determination of phenolic compounds:

To assess phenolic content, 1 g fresh plant sample was homogenized in 10 ml 80% ethanol and agitated for 15 min. at 70°C. One ml of the extract was added to 5 ml of distilled aqueous and 250 µl of 1 N Folin-Ciocalteu reagent and the solution was kept at 25°C. The absorbance was measured with a spectrophotometer at 725 nm. Catechol was used as a standard. The amount of phenolic content was expressed as phenol equivalents in mg/ gm fresh tissue (Saikia *et al.*, 2006).

Statistical analysis

All experiments were performed twice. Analyses of variance were carried out using MSTAT-C program version 2.10 (1991). Least significant difference (LSD) was employed to test for significant difference between treatments at P≤0.05 (Gomez and Gomez., 1984).

RESULTS

Isolation of the associated fungi:

The 16 isolates obtained from chickpea plants were identified as *Fusarium oxysporum* in Mycological Research and Diseases Survey Department, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt.

Results recorded in Table (1) and illustrated in Fig. (1) indicate that among the sixteen isolates, colonies of nine isolates were observed showing compact mycelium while 6 exhibited aerial mycelia. Variations in the mycelium color were observed in the isolates grown PDA medium and were recorded. Initially the color of all isolates was white which changed gradually with different pigments like pinkish white in isolates of *Fo5*, *Fo7*, *Fo10*, *Fo12* and *Fo13*,

pink color was observed in isolates *Fo8*, *Fo6*, *Fo16* and *Fo4*, magenta red turning violet was recorded in isolates *Fo11* and *Fo1*, *Fo14*, *Fo2*, *Fo3* and *Fo9* exhibited white color. Sporulation of isolates showed moderate to profuse. Profuse spores produced isolates from *Fo7*, *Fo8*, *Fo2*, *Fo3* and *Fo9* while isolates *Fo14*, *Fo15*, *Fo1*, *Fo5*, *Fo6*, *Fo11*, *Fo10*, *Fo16*, *Fo4*, *Fo12* and *Fo13* produced moderate spores. The radial growth of colony diameter was differed for different isolates. The colony diameter ranged from 52 to 88 mm for isolates grown seven days after inoculation at 26 °C in 90 mm plates. The maximum radial growth was found in isolates of *Fo13* (88mm) followed by *Fo3* (87mm). Least growth was found in *Fo15* isolate with diameter 52mm.

Table (1): cultural characteristics of *F. oxysporum* isolates grown on PDA medium under *in vitro* conditions.

Location	Isolate code	Colony character	Pigmentation	Sporulation	Diameter (mm) ^a
Assiut	Fo7	Compact Mycelium	Pinkish White	Profuse	80
Assiut	Fo8	Compact Mycelium	Pink	Profuse	82
Assiut	Fo14	Aerial Mycelium	White	Moderate	79
Assiut	Fo15	White fluffy	Pale Yellow	Moderate	52
Beheira	Fo1	Compact Mycelium	Magenta-red turning violet	Moderate	72
Beheira	Fo2	Aerial Mycelium	White	Profuse	84
Beni-Suef	Fo5	Compact Mycelium	Pinkish white	Moderate	74
Beni-Suef	Fo6	Compact Mycelium	Pink	Moderate	76
Gharbia	Fo11	Compact Mycelium	Magenta-red turning violet	Moderate	75
Giza	Fo3	Aerial Mycelium	White	Profuse	87
Minia	Fo9	Aerial Mycelium	White	Profuse	78
Minia	Fo10	Aerial Mycelium	Pinkish White	Moderate	80
New Valley	Fo16	Compact Mycelium	Pink	Moderate	83
Nubaria	Fo4	Compact Mycelium	Pink	Moderate	82
Sharkia	Fo12	Compact Mycelium	Pinkish white	Moderate	80
Sharkia	Fo13	Aerial Mycelium	Pinkish White	Moderate	88

^{a)} DAI =days after inoculation

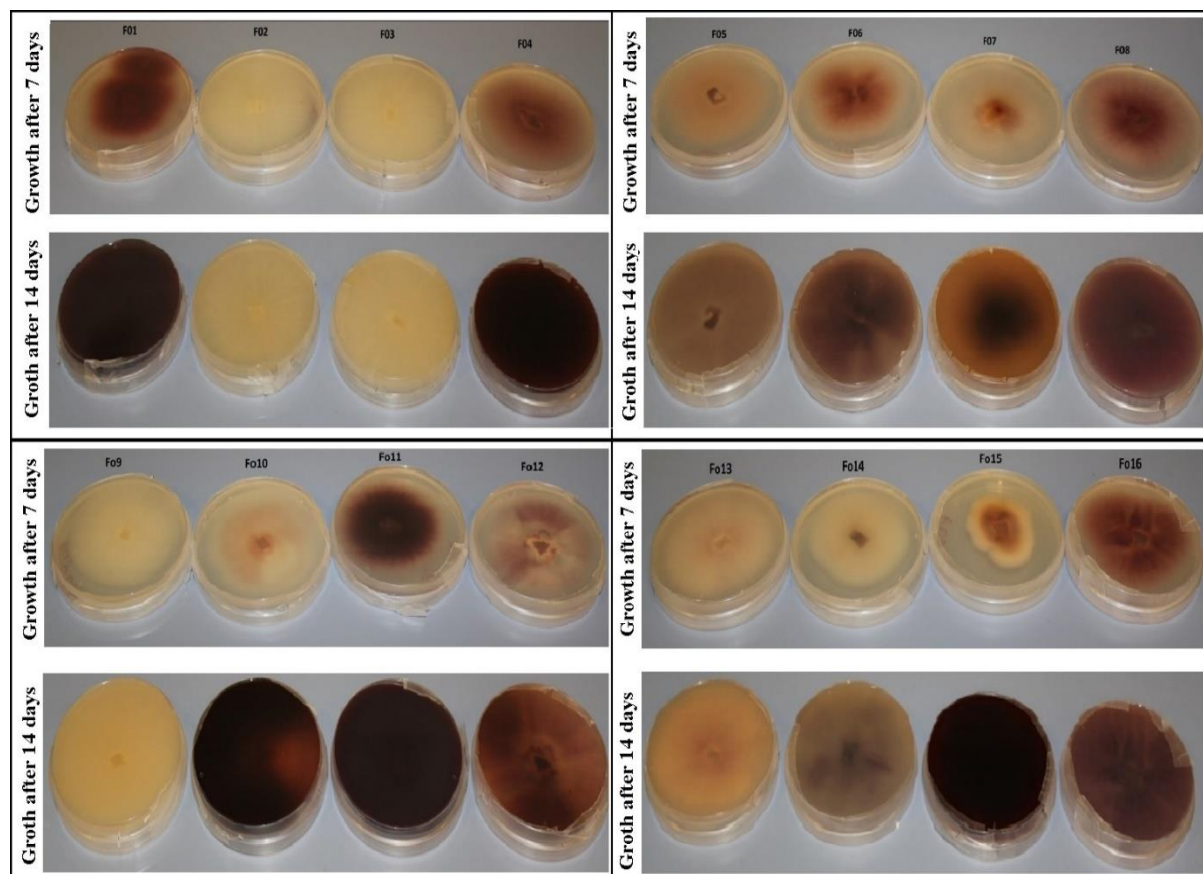


Fig. 1 Mycelial growth of *F. oxysporum* isolates after 7 and 14 days.

Table (2). Pathogenicity test of *F. oxysporum* isolates on chickpea cv. Giza-1 under greenhouse condition and accession number of gene bank.

Location	Cod No	Wilted plants(%)		Survival %	Disease Severity (%)	Virulence degree	Accession numbers
		Early wilt	Late wilt				
Assiut	Fo7	13.30	60.00	26.70	3.50	HV	KY775615
Assiut	Fo8	22.20	43.30	34.50	3.25	HV	KY775616
Assiut	Fo14	30.00	23.30	46.70	2.75	MV	KY775622
Assiut	Fo15	36.70	10.00	53.30	2.50	MV	KY775623
Beheira	Fo1	10.00	23.3	66.70	1.0	LV	KY775609
Beheira	Fo2	20.00	20.00	60.00	2.0	MV	KY775610
Beni-Suef	Fo5	23.30	6.70	70.00	1.0	LV	KY775613
Beni-Suef	Fo6	20.00	3.30	76.70	1.5	MV	KY775614
Gharbia	Fo11	4.40	36.70	58.90	2.0	MV	KY775619
Giza	Fo3	10.00	13.30	76.70	1.5	MV	KY775611
Minia	Fo9	26.70	10.00	63.30	2.0	MV	KY775617
Minia	Fo10	30.0	30.0	40.00	3.25	HV	KY775618
New Valley	Fo16	26.70	20.00	53.30	2.5	MV	KY775624
Nubaria	Fo4	6.7	13.3	80.00	1.0	LV	KY775612
Sharkia	Fo12	16.70	10.00	73.30	1.0	LV	KY775620
Sharkia	Fo13	16.70	23.30	60.00	2.0	MV	KY775621
L.S.D. at 0.05		9.03	7.72	11.51	-	-	-

Sixteen isolates of *F. oxysporum* were evaluated their pathogenicity the susceptible chickpea cultivar (Giza-1) under favorable greenhouse conditions. Results given in Table (2) show that all isolates were pathogenic to the tested cultivar at different levels. All tested isolates caused wilt symptoms on chickpea plants when compared with the control. Isolate No. *Fo15* (isolated from Assiut) caused the highest percentage (36.7 %) of early wilt. While isolate No. *Fo11* (isolated from Gharbia) caused the least percentage of (4.4%) early wilt to the inoculated plants. On the other hand, in case of late wilt disease, isolate No. *Fo7* (isolated from Assiut) caused the highest percentage of late wilt (60 %), while isolate No. *Fo6* (isolated from Beni-Suef) caused the least percentage (3.3 %) of late wilt.

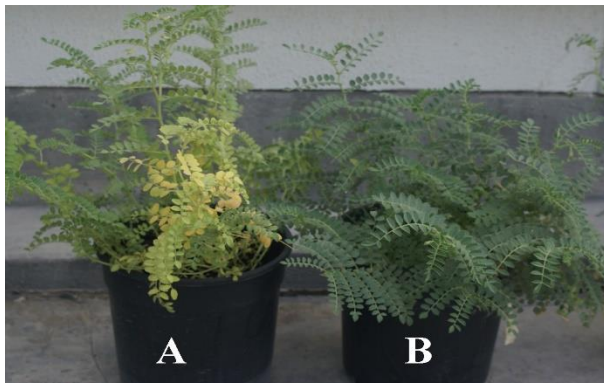


Fig. (2): A- susceptible chickpea cultivar (Giza-1) inoculated with *F. oxysporum* wilted and foliar yellowing syndrome's susceptible reaction; B- healthy (Giza-1) plant uninoculated.

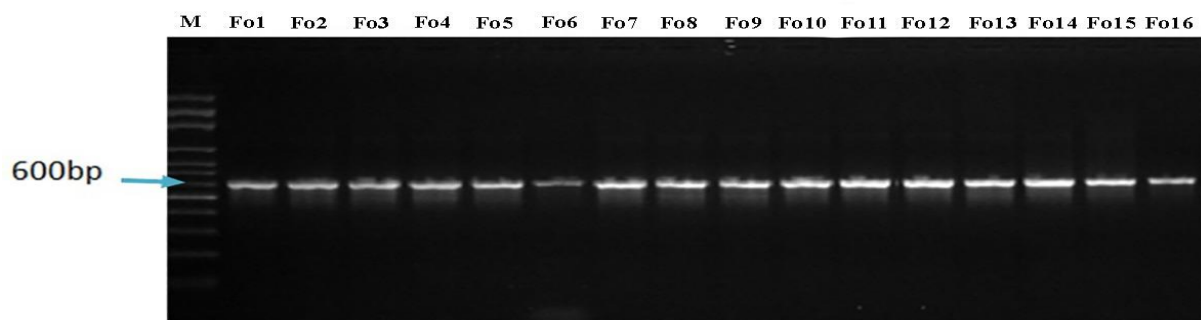


Fig (3): ITS rRNA gene (~600 bp) of the 16 *Fusarium* isolates amplified with universal primers. The amplicon was electrophoretically resolved on a 1.5% agarose gel in 1X TBE buffer Lane1: 100bp DNA plus ladder, Lane F1-F16

Phylogenetic analysis of ITS region:

The phylogenetic tree of sixteen *Fusarium* isolates based on ITS rRNA sequences constructed using MEGA5.2 program are in Fig (4). The phylogenetic tree showed that the sequences can be divided into two main Groups; the first Group divided into two sub Groups; one sub-Group (A) contained seven isolates (*Fo2*,

Concerning disease severity and virulence degree. The results obtained in this study Table (2) indicate that the isolates of *F. oxysporum* the ability to cause disease in susceptible Giza1 chickpea cultivar. The isolates of *Fo7*, *Fo8*, and *Fo10* with various virulence degrees collected from Assiut and Minia regions had high degrees of virulence (HV), isolates *Fo1*, *Fo5*, *Fo4* and *Fo12* were low virulence (LV), and other isolates had moderate degrees of virulence (MV). Furthermore, results showed that *F. oxysporum* isolates with different virulence were detected in all geographic locations and apparently, virulence could not be related to geographic location.

Molecular identification:

PCR ITS amplification :

PCR products of approximately 600 bp amplified with the ITS- F and ITS-B-R primers and corresponding to the ribosomal RNA gene were obtained from all isolates (16 selected isolates) (Fig. 3). After purification of PCR products and sequencing, the BLAST-n alignments results showed that all 16 sequences were associated with high levels of sequence similarity with the ribosomal RNA gene sequences for the *F. oxysporum*.

Sequences were deposited in the GenBank database of 16 accessions number *i.e.*, KY775609, KY775610, KY775611, KY775612, KY775613, KY775614, KY775615, KY775616, KY775617, KY775618, KY775619, KY775620, KY775621, KY775622, KY775623, and KY775624.

Fo5, *Fo8*, *Fo7*, *Fo13*, *Fo6* and *Fo9*). The other sub-Group (B) contained tow isolate (*Fo3* and *Fo15*). While, the second Groups divided into two sub-Groups; one sub-Group (C) contained three isolates (*Fo1*, *Fo11* and *Fo14*). The other sub-Group (D) contained four isolates (*Fo16*, *Fo10*, *Fo4* and *Fo12*).

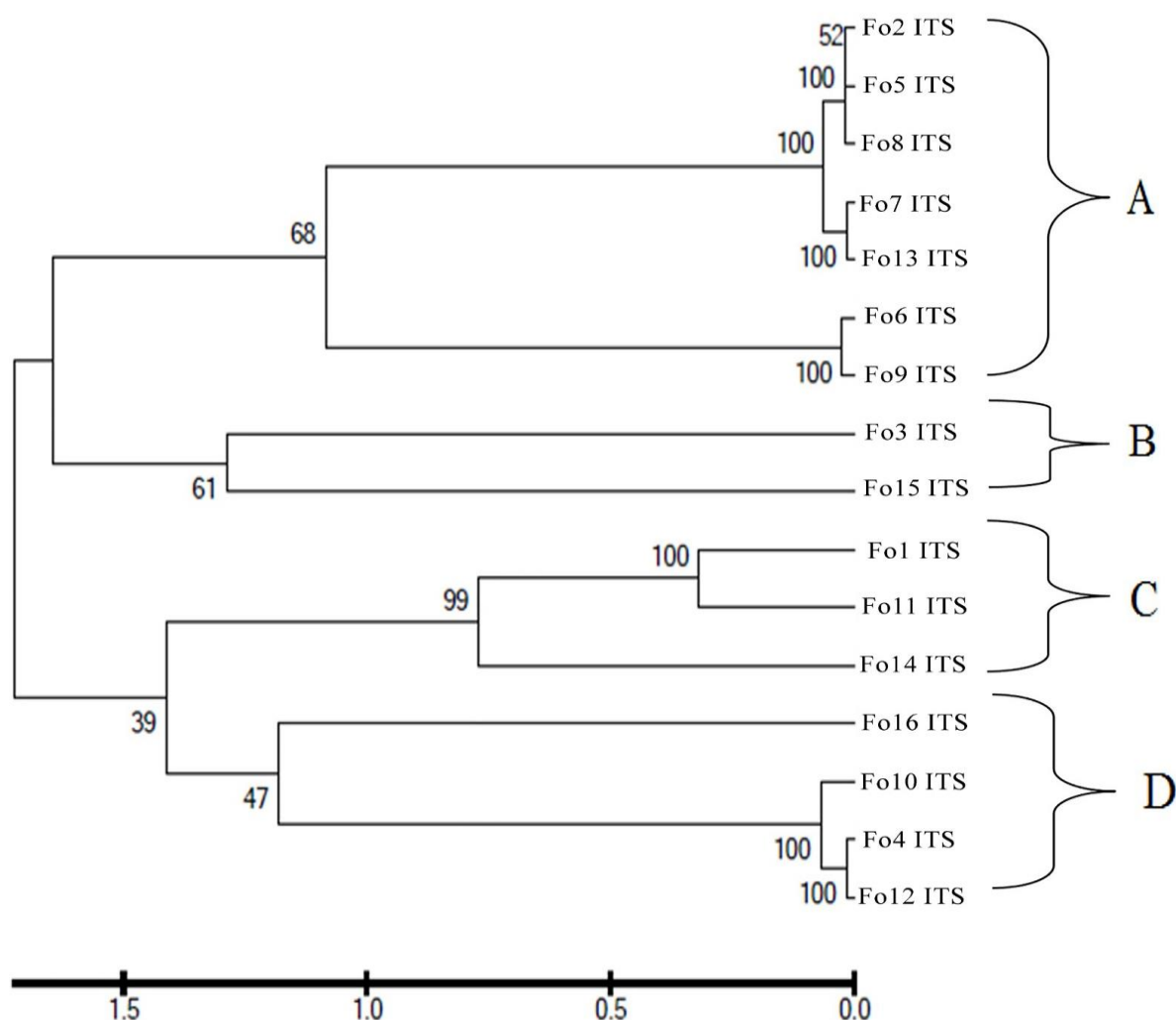


Fig. (4): Phylogenetic tree based on partial ITS rDNA sequences, showing the relationship between 16 *Fusarium* isolates.

Table (3). Reaction of chickpea cultivars against the highly pathogenic *F. oxysporum* isolate (Fo7) under greenhouse conditions.

Cultivars	Early wilt (%)	Late wilt (%)	Survival (%)	Reaction (R) *
Giza-2	6.66	40.00	53.34	M
Giza-195	20.0	56.66	23.34	S
Giza 531	6.66	33.34	60.00	M
Giza-1	13.33	56.67	30.00	S
Giza-88	13.33	46.67	40.00	S
LSD at 0.05	5.62	8.18	14.53	-

(R) * = Resistant (0–20% wilted plants); M = Moderately susceptible (21–50% wilted plants); S = Susceptible > 50% wilted plants

Reaction of chickpea cultivars against the highly Pathogenic isolate of *F. oxysporum* (Fo7)

under greenhouse conditions studied (Table,3). It was observed that Giza-2 and Giza 531 were moderately infected showing early wilt of 6.66%. The survival rates were 53.34% and 60.00%, respectively. Cultivar Giza-195, Giza-1 and Giza-88 were highly susceptible to the pathogen with survival rates 23.34%, 30.00% and 40.00%, respectively (Table,3).

Biochemical analysis:

Peroxidase activity:

The results of peroxidase activity are illustrated in Fig. (5). An increase in peroxidase activity occurred in all chickpea cultivars inoculated with *F. oxysporum* compared with non-uninoculated (healthy). Moreover, the peroxidase activity was higher in moderate chickpea cultivars (Giza-531 and Giza-2) than susceptible ones (Giza-195, Giza-1 and Giza-88). in all cultivars examined, peroxidase activities decreased by increasing the inoculation time from 2 and 5 days to 8 days.

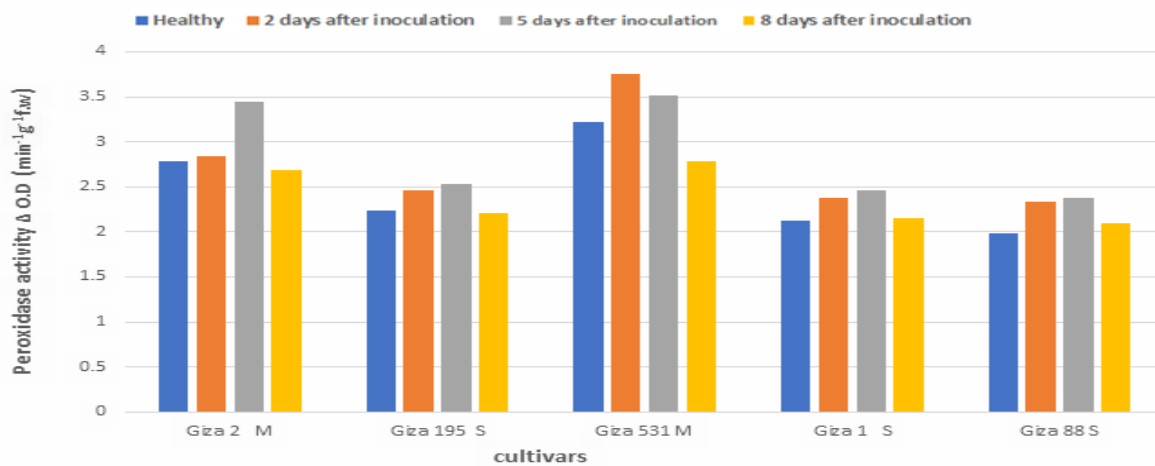


Fig. (5): Peroxidase activity (enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) in chickpea resistant cultivars (Giza-2 M and Giza-531 M), moderate resistant, (Giza-195 S, Giza-1 S and Giza-88 S) and susceptible cultivars inoculated with *F. oxysporum* and uninoculated (healthy) after inoculation time from 2 and 5 day to 8 days.

Polyphenoloxidase activity:

The enzymatic activity of polyphenoloxidase was increased in all the cultivars of chickpeas inoculated with *F. oxysporum* compared with control (Fig. 6). In moderately resistant cultivars, Giza-2 the highest enzymatic activity was recorded after 2 days (0.13 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) followed by Giza-531 (0.12

enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) as compared to susceptible cultivars Giza-195 (0.10 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) followed by Giza-1 (0.09 enzyme unit $\text{min}^{-1}\text{g}^{-1}$) and Giza-88 (0.08 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$). The polyphenol oxidase activity started declining after 2 days in all tested cultivars.

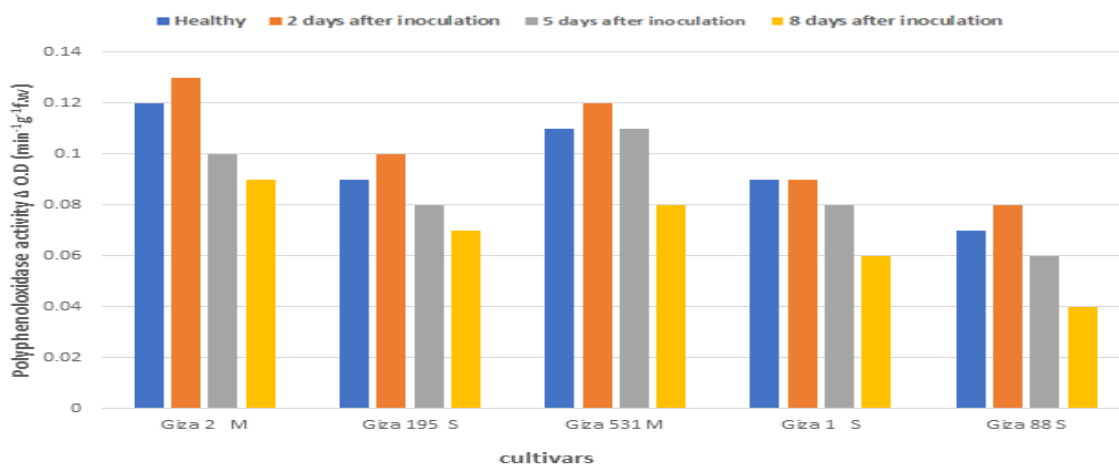


Fig. (6): Polyphenoloxidase activity (enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) in chickpea resistant cultivars (Giza-2 M and Giza-531 M), moderate resistant, (Giza-195 S, Giza-1S and Giza-88 S) and susceptible cultivars inoculated with *F. oxysporum* and uninoculated (healthy) after inoculation time from 2 and 5 day to 8 days.

Catalase Activity:

Results in Fig. (7) show that enzymatic activity of catalase was increased in all the chickpeas cultivars inoculated with *F. oxysporum*. Moderately resistant cultivars, Giza-2 showed the highest enzymatic activity after 8 days (3.86 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) followed by Giza 531 (3.68 $\mu\text{M}/\text{min}$), whereas susceptible

cultivars Giza-195 (1.34 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$), Giza-1 (1.28 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) and Giza-88 (1.16 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) showed the lowest enzymatic activity. The catalase activity continued to increase after 2 days of inoculation in all the cultivars of chickpea examined (Fig.7).

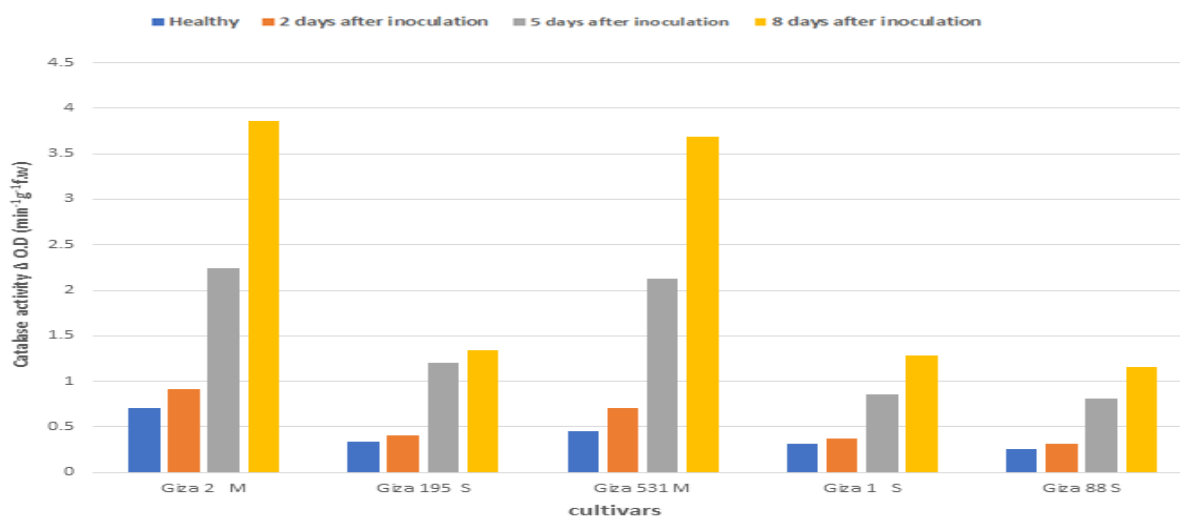


Fig. (7) Catalase activity (enzyme unit min⁻¹g⁻¹f.w.) in chickpea resistant cultivars (Giza-2 M and Giza-531 M), moderate resistant, (Giza-195 S, Giza-1 S and Giza-88 S) and susceptible cultivars inoculated with *F. oxysporum* and uninoculated (healthy) after inoculation time from 2 and 5 day to 8 days.

Phenylalanine ammonia lyase

There was an increase in the activity of PAL in all the cultivars when inoculated with *F. oxysporum*. The obtained data recorded in fig. (8) indicate that moderately resistant cultivars Giza-2 recorded the highest enzymatic activity after 8 days (5.23 enzyme unit min⁻¹g⁻¹f.w.) followed by Giza 531 (5.21 enzyme unit min⁻¹g⁻¹f.w.). The PAL enzymatic activity was less in

susceptible cultivars Giza-195 (2.14 enzyme unit min⁻¹g⁻¹f.w.) followed by Giza-1 (2.08 enzyme unit min⁻¹g⁻¹f.w.) and Giza-88 (1.56 enzyme unit min⁻¹g⁻¹f.w.) as compared to moderately resistant cultivars. The Phenylalanine ammonia lyase activity continued to increase after 2 days of inoculation in all the chickpea cultivars.

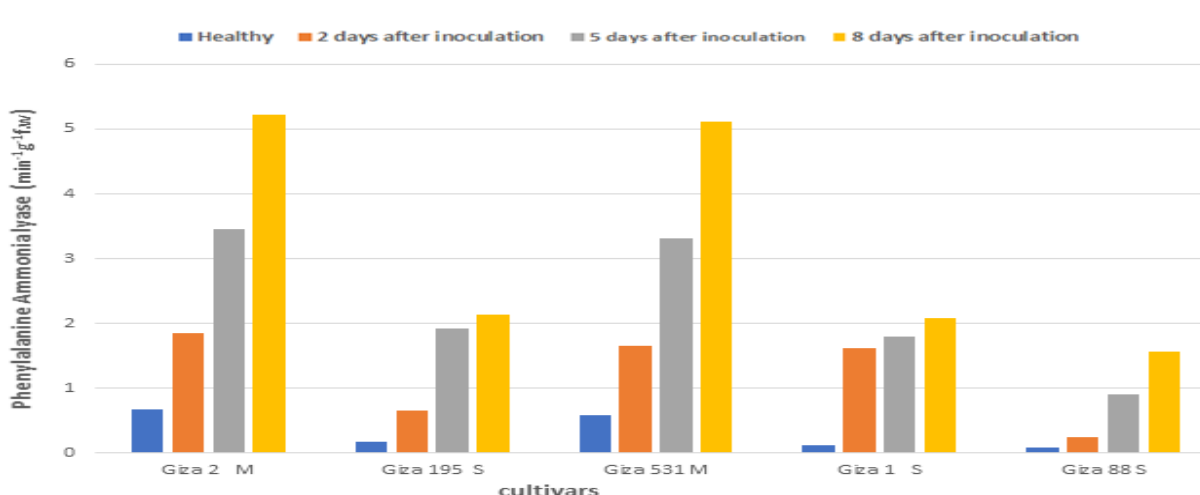


Fig. (8) Phenylalanine Ammonia lyase (enzyme unit min⁻¹g⁻¹f.w.) in chickpea resistant cultivars (Giza-2 M and Giza-531 M), moderate resistant, (Giza-195 S, Giza-1 S and Giza-88 S) susceptible cultivars inoculated with *F. oxysporum* and uninoculated (healthy) after inoculation time from 2 and 5 day to 8 days.

Phenolic compounds

Data present in Fig. (9) show that phenolic compounds increased in all the cultivars of chickpea when inoculated with *F. oxysporum* compared to the control uninoculated. The highest phenolic compounds were recorded after

5 days in moderately resistant cultivar Giza-2 (1.46 mg/mg f.w.) followed by Giza 531 (1.38 mg/gm f.w.). Whereas the lowest TPC were recorded in susceptible cultivar Giza-195 (0.86 mg/gm f.w.) Giza-1 (0.74 mg/gm f.w.) and Giza-88 (0.68 mg/gm f.w.).

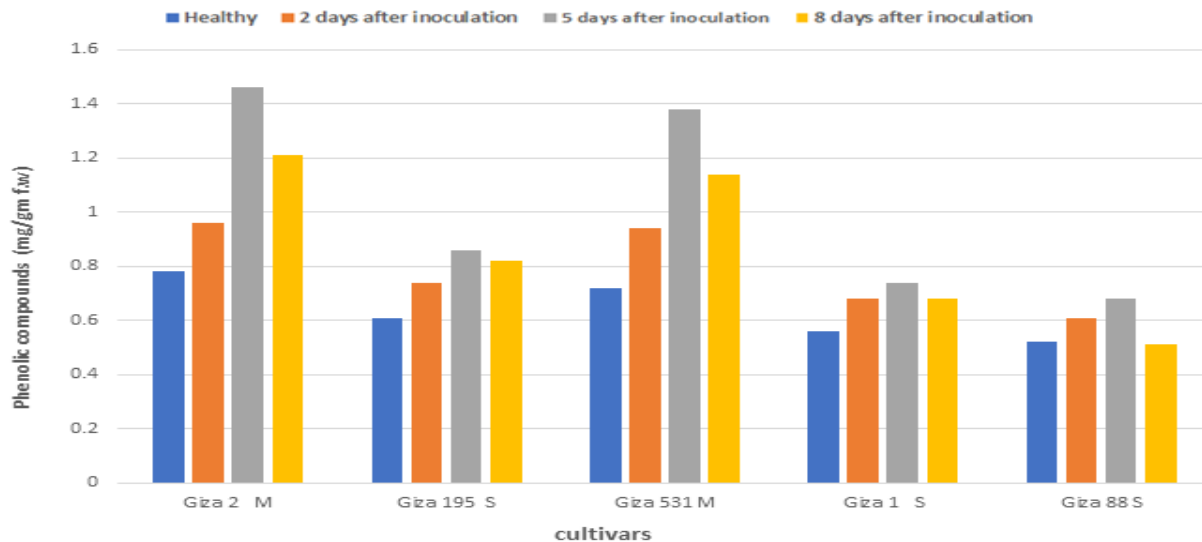


Fig. (9). Total phenolic compounds (mg/gm f.w.) in chickpea resistant cultivars (Giza-2 M and Giza-531 M), moderate resistant, (Giza-195 S, Giza-1 S and Giza-88 S) susceptible cultivars inoculated with *F. oxysporum* and uninoculated (healthy) after inoculation time from 2 and 5 day to 8 days.

DISCUSSION

The importance of this investigation is to shed light on morphological and molecular techniques used to identify some *Fusarium* species isolated from chickpea and assessment of virulence of these isolates, as well as identify the different resistant sources in chickpea cultivars.

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

F. oxysporum in this study were variable in their colony characters, pigmentation, sporulation degree and radial growth. These results are compatible with the results recorded by Dubey *et al.* (2010) and Arvayo-Ortiz *et al.*, (2011).

The present study showed a positive relation between the amount of spore production and virulence of isolates. The isolates produced abundant sporulation were highly virulent, while moderately sporulated isolates produced moderately virulent or low virulent (Golakiya *et al.*, 2018).

The molecular characterization can also be a useful tool to phylogenetically relate the fungi on the basis of their characteristic morphological features. The current results indicated that molecular identification of the organisms exhibited high specificity and sensitivity and can be used for classifying microorganisms at taxonomical level.

Reproduction of ITS regions ensured the definition of relationships between relatives of distant taxa. Thus, detailed information on genetic characteristics of the isolates studied has been obtained. The information obtained in such studies is crucial in solving the taxonomic problems of the genera that will be studied based on molecular phylogenetic and revealing the position at molecular level in fungi systematic.

Similarity index and dendrogram data obtained in the genetic characterization study of *Fusarium* spp. revealed the genomic similarities and differences of chickpea fungal isolates.

The phylogenetic tree obtained revealed that strains had the highest homology with *Fusarium*, the virulence of these isolates was between low virulent and highly virulent. The results concluded that *Fusarium* isolates with a high

virulence (HV) were detected in Assiut and Minia, while the remaining isolates appear to have between moderately virulent (MV) and low virulent (LW).

Resistant cultivars are the most practicable, feasible, and economical approach for the management of wilt disease of chickpea, but only a few sources with low level of genetic resistance are available, so there is a need to identify the resistant sources in chickpea. Biochemical analysis is important to understand the resistant mechanism before and after infection of plant and to isolate the resistance sources (El-Awad., 1993 and Salme *et al.*, 1991). According to our results, 2 cultivars were found moderate resistant were Giza-2 and Giza-531, while cultivar Giza-195, Giza-1 and Giza-88 were susceptible ones.

Oxidative enzymes *i.e.*, peroxidase and polyphenoloxidase were considered in shoot samples of susceptible cv. Giza-195, Giza-1, Giza-88 and moderately resistance cv. Giza-2, Giza-531 after 2, 5 and 8 days of inoculation with *F. oxysporum*. The obtained results showed that infected chickpea plants had a higher peroxidase and polyphenoloxidase activities than the healthy ones. Also, the moderately resistant cultivar had higher peroxidase and polyphenoloxidase activity than the susceptible one. These differences in enzymatic activities might be attributed to that oxidative enzyme play a partial role in activating inducible defense of plant (Vera-Fstrella, *et al.*, 1994). The high increment in enzymatic activity happened in early stages in moderately resistance cultivar and hindered infection progress successfully, and by time, that enzymatic activity retreated to the normal levels (like in the healthy). While, on the contrary, in the susceptible cultivar enzymatic activity increased gradually with the time after inoculation but with low levels and failed to hinder the infection progress. These results are compatible with the results recorded by Dalvi *et al.* (2011). There was an increase in the activity of peroxidase in resistant cultivars than susceptible ones to *F. oxysporum* f. sp. *ciceri* inoculated seedlings over control. In Vijay (wilt resistant), peroxidase activity was increased from 2.06 to 8.54 units (un-inoculated over inoculated) and in JG-62 (susceptible) activity was increased from 1.68 to 1.96 units (un-inoculated over inoculated) in seedlings. Moreover, enhanced peroxidase activity was linked with synthesis of lignin (Ride., 1975).

There was an increase in the activity of catalase in all the cultivars when inoculated with *Fusarium oxysporum*. Highest enzymatic activity was recorded after inoculation in moderately resistant cultivar Giza-2 (3.86 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) which the highest enzymatic activity after 8 days followed by Giza-531 (3.68 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) Whereas, the activity in susceptible cultivars Giza-195 (1.34 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$), Giza-1 (1.28 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) and Giza-88 (1.16 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) showed the less enzymatic activity.

Catalase activity increased during infection as a mechanism to scavenge fungi toxic H_2O_2 . The major function of catalase within cells is to prevent the accumulation of toxic levels of hydrogen peroxide formed as a by-product of metabolic processes primarily that of the electron transport pathway (Montalibini, 1991).

The increase in the activity of PAL in all the cultivars was recorded when chickpea was inoculated with *F. oxysporum*. moderately resistant cultivars Giza-2 recorded the highest enzymatic activity after 8 days (5.23 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) followed by Giza-531 (5.12 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$). The PAL enzymatic activity was less in susceptible cultivars Giza-195 (2.14 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) followed by Giza-1 (2.08 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) and Giza-88 (1.56 enzyme unit $\text{min}^{-1}\text{g}^{-1}\text{f.w.}$) as compared to moderately resistant cultivars. Therefore, one can conclude that the plant defense mechanism started after the pathogen attack with higher synthesis of Phenylalanine ammonia-lyase PAL compounds and defense related enzymes. Phenylalanine ammonia-lyase (PAL) was the main enzyme of plant phenolic production including those under stress exposure. It was the key enzyme that catalyzed core reaction in phenylpropanoid metabolism leading to functionally diverse defense related products such as lignin, suberin, wall bound phenolic and flavonoids (Wen *et al.*, 2005). It is well known that phenolic compounds are now to impart resistance against fungal diseases. This could be explained on base of the fact that phenolic compounds are toxic to several plant pathogens. Also, the active ingredients of some fungicides are of radical of phenolic compounds (Vidhyasekaran, 2004). Consequently, recorded higher level of phenolic compounds in the moderately resistance cultivar Giza-2, Giza-531 support evidence of the role of the phenolic compounds against the chickpea *Fusarium* wilt.

CONCLUSION

The present investigation gave a set of information about the *F. oxysporum* isolates in terms of biodiversity both in morphological and genetically background, their geographical distribution, pathological ability as well as metabolic changes in resistant and susceptible chickpea cultivars. Therefore, cooperation between pathologists and plant breeders seeking for breeding chickpea resistant to wilt disease is highly required as a safe, economic and stable mean of disease control program.

CONFLICTS OF INTEREST:

The authors declare no conflict of interest exists.

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