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Evaluation and Characterization of Some Egyptian *Fusarium oxysporum* Isolates for their Virulence on Tomato and PCR Detection of (SIX) Effector Genes

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Abstract

The genus Fusarium is comprises of different species which are furtherer divided into small groups called *formae speciales* according to their host specificity. It is remarkable that while an isolate belonging to particular *formae speciales* is considered highly pathogenic in a certain plant species, the many other isolates belonging to the same *formae speciales* may have no harm effect or even a beneficial relation to the same host plant. Traditionally, bioassays conducted under green house conditions were used to distinguish pathogenic and non-pathogenic isolates. However, such these techniques have some limitations due to the time consuming and the inconsistency of obtained results. In contrary, molecular identification techniques are candidate to play an important role in the characterization process of Fusarium isolates as well as with many other organisms. Recently, the ability of a Fusarium isolate to infect particular plant species was found to be dependent on specific genes encoding host determining 'virulence factors' that distinguish virulent from avirulent strains. More recently, eight fungal proteins were identified from xylem sap of infected plants, encompassing the small secreted proteins called Six1, Six2, Six3, Six4, Six5, Six6, Six7 and SIX 8. In the present study, the linkage between virulence potential of fifteen pathogenic isolates in addition to one non-pathogenic isolate, Fo162 , of *Fusarium oxysporum* and the presence of secreted in xylem effector genes (SIX) was determined. The results showed that the tested isolates were varied regarding to their pathogenicity potential toward tomato plants under greenhouse conditions. On the other hand, the amplicons of SIX1, SIX5 and SIX 7 were detected with most hyper virulent isolates while no amplicons for any tested SIX genes were observed with the non-pathogenic isolate (Fo162) as well as with the most hypo virulent isolates. These results, suggested that SIX1, SIX5 and SIX6 may play a distinct role in virulence potential of these *Fusarium oxysporum* isolates toward tomato plants under Egyptian conditions.

Keywords: *Fusarium oxysporum*; Virulence; Effector genes; PCR

Introduction

Tomato (*Solanum lypersicum* Mill.) is ranked number 1 among fruits and vegetables with 14% of the total production worldwide. World production of tomato covers approximately 4 million hectares of arable land with production estimated at 100.5 million tons and valued at 5-6 billion US$ [1]. Note worthy, Egypt is among the top 10 tomato producing countries worldwide with estimated 181.000 ha of harvested area which producing about 6.4 million tons [1].

Of all vegetable crops, tomato in particular, is heavily infected with many different plant parasites and pathogens [2]. More than 200 diseases were recorded on tomato plants and were considered responsible for 70 to 95% of the annual losses of tomato production while Fusarium wilt disease is only responsible for 10 to 50% of these losses [3]. In Egypt, the losses due to tomato wilt disease reached up to 67% [4].

Many isolates of *Fusarium oxysporum* are known to be important plant pathogens that cause severe damage to many host plants [5-7]. Moreover, pathogenic strains have been grouped into host-specific forms called *formae speciales* (f.spp.), which are sometimes divided further into races based on cultivar specificity [8-10]. As a result, Fusarium genus contains approximately 120 *formae speciales* and races, based on the plant species and cultivars they infect [8,11,12]. Different isolates of a particular forma speciales are known to be varied in their virulence potential toward a certain plant species [5,12-15].

Generally, virulence characterization achieved by traditional pathogenicity bioassays under greenhouse conditions and recently through molecular approaches. Molecular identification techniques are candidate to be among the most promising useful tolls for Fusarium isolate identification as well as for many other pathogens due to the accuracy in addition to the consistency of the results [16,17].

The ability of a Fusarium isolate to infect particular plant species depends on specific genes that distinguish virulent and avirulent strains. Recently, 'virulence factors', that encoding host determining small secreted proteins, called effectors, are found to play a crucial role in Fusarium virulence potential on his hosts [18]. During the preliminary infection stage, Fusarium isolates secret a special protein and inject it into the xylem of the host plant leading to initiation of infection process. On the other hand, in resistant host, immune plant genes (i-) can recognize and counter-attack the effect of these virulence effectors [19].

In order to control this disease in tomato plants, different approaches including obtaining resistant cultivars and good understanding of pathogen population specially its virulence genes and their effects. Therefore, 15 different *Fusarium oxysporum* isolates were selected to present delta area in Egypt as a start for studding the Egyptian *Fusarium oxysporum* population.

Recently, in tomato several in planta secreted proteins have been identified with *F. oxysporum* f. sp. *lycopersici* (Sacc.). The first identified secreted in xylem protein (SIX) with *Fusarium oxysporum* f.sp. *Lycopersici* was called (Six1), which is a small cysteine-rich protein

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required for full virulence on tomato [20]. In addition, recognition of the protein by tomato plants carrying the resistance gene I-3 leads to disease resistance as mentioned by Rep et al. [21]. Therefore, Six1 is also called Avr3 to indicate its gene-for-gene relationship with the I-3 resistance gene. More recently, additional fungal proteins were identified from xylem sap of infected plants, encompassing the small secreted proteins Six2, Six3, Six4, Six5, Six6, Six7 and SIX 8 [22,23].

This study is aimed to:

1. Evaluation of virulence potential of 15 different Egyptian isolates of *Fusarium oxysporum* toward tomato using pathogenicity bioassay under Egyptian greenhouse conditions.

2. Characterization of these isolates for presence and absence of genes SIX1, SIX2, SIX3, SIX4, SIX5, SIX6, SIX7 and SIX8 using molecular technique based on PCR polymorphism analysis.

Materials and Methods

Fungal isolates

Fungal isolates of *Fusarium oxysporum* were originally isolated during 2013-2014 seasons from the cortical tissues of surface sterilized roots of naturally infected tomato plants (*Lycopersicum esculentum* Mill.) showed the typical symptoms of Fusarium wilt disease and grown in five different provinces in Egypt (Table 1). Fungal isolates which had culture and microscopic characteristics corresponding to those isolates of *Fusarium oxysporum* were cultured on Potato Dextrose Agar (PDA) media amended with 150 mg^{-1} streptomycin and 150 mg^{-1} chloramphenicol and purified using single spore colony technique. Purified isolates were reared on PDA plates and incubated at 27°C in the dark for 14 days. For each isolate, several small disks (≈1 mm in diameter) were cut using a sterilized cork borer and stored in micro-bank tubes at -80°C. These initial disks were used later for preparing the inoculum for using in the next investigations.

Fungal inoculum for pathogenicity test

For preparation of the fungal inoculum for pathogenicity test in greenhouse, the initial disks of Fusarium isolates were cultured on PDA plates for 2 weeks. The mycelia and spores were scratched from the surface, suspended in water, sieved through 3 layers of cheese cloth and number of spores was counted using a Haemocytometer slide (Thoma, Germany). The concentration was adjusted using sterilized tap water. Seeds of "Super Marmande`` tomato cultivar which is susceptible to Fusarium wilt disease were sown in plastic trays filled with autoclaved beetmooth substrate (company, Egypt). After 45 days, seedlings were transplanted in plastic pots filled with 3 kg of soil and sand mixture (1:2, w/w). Two days after transplanting, tomato seedlings were injected 2 cm deep into the rhizosphere using 3 holes made around the stem base with 2×10^6 conidia suspended in 2 ml water. Five replicates were used within each Fusarium isolate. Control plants were treated only with 2 ml of tap water. Disease incidence and disease severity were recorded after 4 weeks from pathogen inoculation according to Vakalounakis and Fragkiadakis [24] on 0 to 3 visual scales:

0= no symptoms

1= light yellowing of leaves, light or moderate rot on tap root and secondary roots and crown rot

2= moderate or severe yellowing of leaves with or without wilting, stunting, sever rot on taproot and secondary roots, crown rot with or without hypocotyls rot, and vascular discoloration in the stem

3= Dead plants.

Disease severity was determined using the following formula:

$$Disease\ severity(\%) = \left[\frac{\left(\sum scale \times number\ of\ plants\ \text{inf}\ ected\right)}{Highest\ scale \times total\ number\ of\ plants}\right]$$

DNA extraction

For isolating the DNA from *Fusarium oxysporum* isolates, 1 disk of each isolate was cultured into 500 ml flask containing 200 ml of potato dextrose broth media (PDB, Defco, Germany) and incubated at 27°C at 100 rpm shaker. After 14 days, the mycelia and spores were sieved through 3 layers of cheese cloth. The mycelium was dried between 2 layers of sterilized filter papers and immediately freeze dried using lyfolization apparatus for overnight. 100 mg of the freeze dried mycelium was subjected into DNA extraction using GE Health care, illustra™, Uk kit (Table 1).

Genetic polymorphism analysis

For investigating the presence and absence of SIX genes, the DNA of 15 isolates of pathogenic *Fusarium oxysporum* isolated from tomato plants grown in five different governorates in Egypt in addition to 1 isolate of non-pathogenic *Fusarium oxysporum*, Fo162, which was kindly provided by Prof.Richard Sikora, Bonn University, Germany, was obtained using the same procedure mentioned above. DNA was diluted 10x proier to PCR amplification. PCR fragments were amplified by PCR using the 8 different primers of secreted in xylem (SIX) genes (Table 2).

The PCR mix contained 10 µL 5X Green Go Taq reaction buffer (Promega), 2 µl dNTPs (10 mM), 1 µl forward primer, 1 µl backward primer, 0.25 µl Tag polymerase (0.625 units) and 37.75 µl water and 2 µl fungal DNA as template. Polymerase Chain Reaction (PCR) procedure was performed in a Bio-RAD, C1000 thermo cycler by an initial denaturation at 95°C for 4 min, followed by 34 cycles of 95°C for 1 min, 50°C for 2 min and 72°C for 3 min. After the PCR procedure, samples were stored at 4°C. For each isolate with each SIX primer, 10 µL of the PCR product were injected into agarose gel electrophoresis to determine the presence and absence of effector avirulence gene.

Isolate number	Governorate	District	location
1	Kafer el-Sheihk	Sedi Ghazy	Sedi Nasr
2	Kafer el-Sheihk	Balteem	Sidi-Mubarak
3	Gharbya	Santa	Balkem
4	Giza	Giza	Agriculture research center
5	Kafer el-Sheigh	Balteem	Al-Shahabiya
6	Kafer el-Sheigh	Sakha	Al-Hamrawy
7	Kafer el-Sheigh	Sedi GHazy	Dukmera
8	Dakhahlia	El-Mansoura	Meet Ali
9	Dakhahlia	Shrbeen	El-Wekala
10	Dakhahlia	Talkha	Batrah
11	Dakhahlia	Meet Ghamr	Damas
12	Kafer el-Sheigh	Sedi Ghazy	Almanshiya
13	Gharbya	Qutuor	Qutur
14	Gharbya	Santa	Al-Gemmeza
15	Sharkiya	Zagazig	Al-Qinayat

Table 1: Pathogen isolates of *Fusarium oxysporum* isolated from infected tomato plants grown in different locations in Egypt and showed the typical symptoms of Fusarium wilt disease during 2013-2014 seasons.

Gel electrophoresis analysis

The agarose gel used in this analysis was prepared with 1 X Tris-Acetate EDTA-Buffer (TAE, AppliChem). 1 g agarose (Sigma) was added to 100 ml of TAE buffer and heated for 5 minutes in a microwave (MW800, Continent) at 650 watts. After cooling at approx. 50°C, 3 µL of 10mg ml^{-1} Ethidium Bromide (AppliChem) was added. This solution was poured into an electrophoresis tray and left for approx. 30 minutes until the gel had solidified. The gel was subsequently transferred to the gel electrophoresis chamber filled with 1 X TAE buffer. After transferring all samples to the wells of the gel, the electrophoresis analysis was conducted for 60 minutes at 80 Volt. An Ultraviolet transilluminator (BIO-RAD, Gel DOC™ –XR+) was used to visualize the DNA bands.

Results

Pathogenicity test

Fifteen isolates which showed the identical morphological and microscopically characters of *Fusarium oxysporum* species were isolated from infected tomato plants grown in fields located in 5 different governorates in Egypt (Table 1). To determine the virulence potential of obtained *Fusarium oxysporum* isolates, pathogenicity test was conducted under greenhouse conditions in Egypt. The results showed that all the tested isolates were pathogenic toward tomato plants and caused symptoms corresponding to the Fusarium wilt disease. Moreover, results revealed that the tested isolates were varied regarding to their aggressiveness and virulence potential under the bioassay conditions (Figure 1). In deed, the results illustrated that the highest percentage of disease severity i.e. 61%; 52%; 44%; and 41.65% were recorded within isolates 4; 5; 15; 2 respectively (Figure 1). On the other hand, isolates 8; 12 and 14 resulted in the lowest disease severity percentage on tomato plants (8,79%; 9,7% and 9,79% respectively)

Molecular identifications

Presence and absence of SIX effector genes: The linkage between virulence potential of fifteen pathogenic isolates in addition to one non-pathogenic isolate, Fo162 , of *Fusarium oxysporum* and the presence of secreted in xylem effector genes (SIX) was determined. The results showed that amplicons of SIX1 were detected with all isolates tested except isolates 8, 12, 14 and 15 in addition to the non-pathogenic isolate, Fo162, (Figure 2 and Table 3). Furthermore, no amplicons for SIX2, SIX3 and Six4 were detected among all *Fusarium oxysporum* isolates subjected to the test (Figures 2 and 3, Table 3). Similar results were also observed with SIX7 and Six8 (Figure 4). In contrarily, amplicons of Six6 were detected within all pathogenic tested isolates except isolates 1, 6, 7, 8, 11 and isolate 12 (Figure 5). Additionally, amplicons of SIX5 were detected only within isolates 2, 3, 4, 5, 7 and 15 (Figure 5, Table 3).

Discussion

Pathogenicity test was conducted under greenhouse conditions to determine the virulence potential of fifteen isolates belonging to species *Fusarium oxysporum*. The obtained results showed that all tested isolates were pathogenic on tomato plants under the bioassay conditions and can be classified into three different groups according to their virulence potential (Table 4). The first group contains the hyper-virulent isolates while the second group contains the moderate virulent isolates and the last group contains the hypo-virulent isolates (Table 4). To verify the bioassay results, the fifteen tested pathogenic isolates of *Fusarium oxysporum* in addition to one non-pathogenic Fusarium isolate, Fo162, were subjected to the polymorphic analysis based on screening the presence and absence of the amplicons of 8 different secreted in xylem genes (SIX1, SIX2, SIX3, SIX4, SIX5, SIX6, SIX7 and SIX8). The obtained results illustrated that the amplicons of the three effectors, Six1, Six5 and Six6, were detected with most isolates of the hyper-virulent group while only two of the Six genes were detected within isolates of the moderate virulent group except isolate 3 which contain the three SIX genes (Table 4). Furthermore, No amplicons for any of the tested SIX genes were detected within any of the isolates

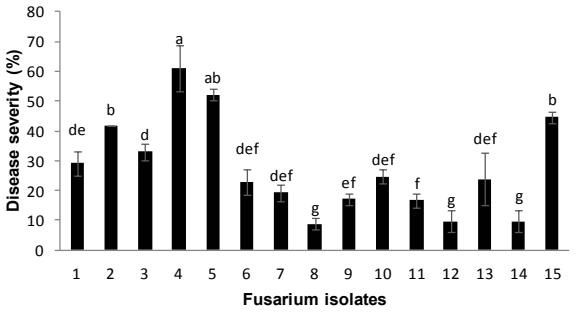

Figure 1: Disease severity recorded after 4 weeks on tomato plants inoculated with 15 different individual isolates of *Fusarium oxysporum* (1:15). Paired Means with different letters are significantly different based on Tukey test ($p \leq 0, 05$; n=5).

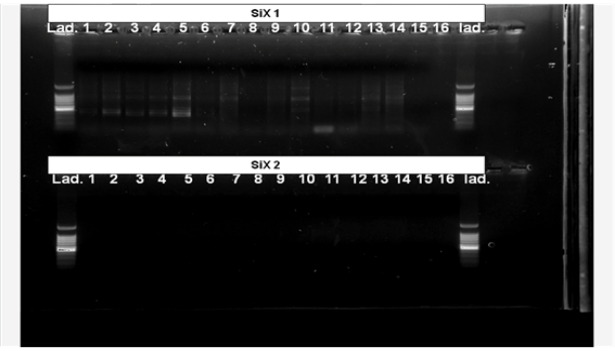

Figure 2: PCR amplicons generated by SIX 1 and SIX 2 primers using genomic DNA of 15 pathogenic isolates (lanes from 1:15) and one non-pathogenic isolate, Fo162, (lan16) of *Fusarium oxysporum*. Lad. is 1Kbp DNA ladder

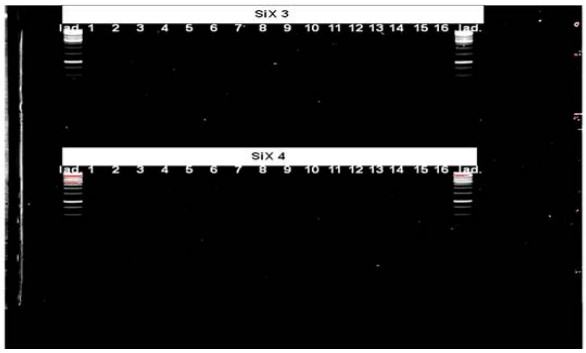

Figure 3: PCR amplicons generated by SIX 3 and SIX 4 primers using genomic DNA of 15 pathogenic isolates (lanes from 1:15) and one non-pathogenic isolate, Fo162, (lan16) of *Fusarium oxysporum*. Lad is 1Kbp DNA ladder.

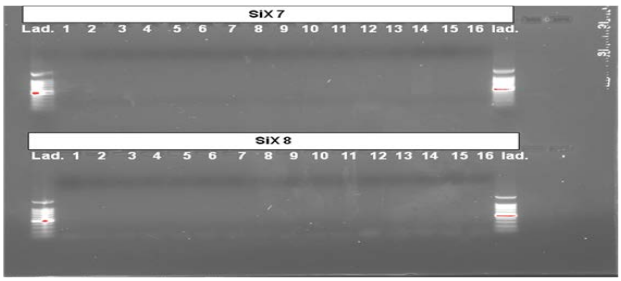

Figure 4: PCR amplicons generated by SIX 7 and SIX 8 primers using genomic DNA of 15 pathogenic isolates (lanes from 1:15) and one non-pathogenic isolate, Fo162, (lan16) of *Fusarium oxysporum*. Lad is 1Kbp DNA ladder.

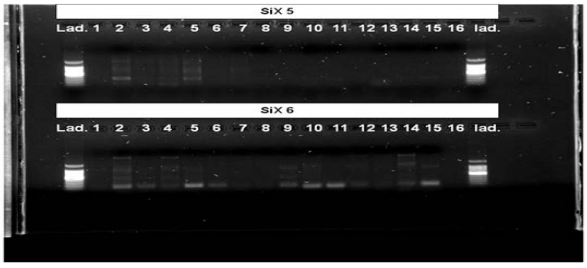

Figure 5: PCR amplicons generated by SIX 5 and SIX 6 primers using genomic DNA of 15 pathogenic isolates (lanes from 1:15) and one non-pathogenic isolate, Fo162, (lan16) of *Fusarium oxysporum*. Lad is 1Kbp DNA ladder.

belonging to the hypo-virulent group except isolate 14. Note worthy, amplicons of genes SIX2, SIX3, SIX4, SIX7 and SIX8 could not be detected within any of the tested isolates which may suggest their absence in the Egyptian isolates.

These results are in consistence with results of Lievens et al. [23] who reported that SIX1, SIX2, SIX3 and SIX5 can be used for the unambiguous identification of *F. oxysporum f. sp. lycopersici* while Six6 and SIX7 were presented in few *Fusarium oxysporum formae speciales* and suggested that these genes may play a more general role in Pathogenicity. In 2002, Rep et al. [25] mentioned that SIX1 gene encodes a protein which was detected in xylem sap of infected tomato plants and this protein is likely to play a role in host colonization at least under some conditions or in some hosts and it was clearly that SIX1 is not required for pathogenicity under the conditions of their bioassay. Furthermore, Rep et al. [21] and Houterman et al. [19] reported that two avirulence proteins of *Fusarium oxysporum*, Avr3 (SIX1) and Avr1 (SIX4), have been recently identified and we found to be required for full virulence of *Fusarium oxysporum* on tomato. Moreover, the virulence function has been established for tow of these small proteins using gene knockout experiments [26]. These are Six4 (Avr1), which is required for I and I-1-mediated resistance [27-29], and Six3 (Avr2), which is required for I-2-mediated resistance while the validity of virulence function of the other SIX genes still under investigation. In addition, Six4/Avr1 was found to suppress I-2- and I-3-mediated disease resistance [27,30,31].

In conclusion, the results obtained from present study suggest that

name	Sequence of forward primer (5 - 3`)	Sequence of backward primer (5 - 3`)
Six1	GTATCCCTCCGGATTTTGAGC	AATAGAGCCTGCAAAGCATG
SIX2	CAACGCCGTTTGAATAAGCA	TCTATCCGCTTTCTTCTCTC
SIX3	CCAGCCAGAAGGCCAGTTT	GGCAATTAACCACTCTGCC
SIX4	TCAGGCTTCACTTAGCATAC	GCCGACCGAAAAACCCTAA
SIX5	ACACGCTCTACTACTCTTCA	GAAAACCTCAACGCGGCAAA
SIX6	CTCTCCTGAACCATCAACTT	CAAGACCAGGTGTAGGCATT
SIX7	CATCTTTTCGCCGACTTGGT	CTTAGCACCCTTGAGTAAC
SIX8	ATGCAACCCCTACGCGTTCT	CTAGAAATTGTTTATAAACTGGAC
FO162	CGAGCAACCTCTCAGTATCAGATC	CGGAGCCTGCAAAGCTGTAC
FO162	CAAACCGGGCGAGCAA	GGAGCCTGCAAAGCTGTACAG

Table 2: Sequences of forward and backword primers of SIX1, SIX2, SIX3, SIX4, SIX5, SIX6, SIX7 and SIX8 genes in addition to specific forward and backword primers for one non-pathogenic *Fusarium oxysprum* isolate (Fo162)

isolate	SIX1	SIX2	SIX3	SIX4	SIX5	SIX6	SIX7	SIX8
1	+	-	-	-	-	-	-	-
2	+	-	-	-	+	+	-	-
3	+	-	-	-	+	+	-	-
4	+	-	-	-	+	+	-	-
5	+	-	-	-	+	+	-	-
6	+	-	-	-	-	-	-	-
7	+	-	-	-	+	-	-	-
8	-	-	-	-	-	-	-	-
9	+	-	-	-	-	+	-	-
10	+	-	-	-	-	+	-	-
11	+	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	+	-	-	-	-	+	-	-
14	-	-	-	-	-	+	-	-
15	-	-	-	-	+	+	-	-
Fo162	-	-	-	-	-	-	-	-

Table 3: PCR-screen targeting the SIX genes, SIX1, SIX2, SIX3, SIX4, SIX5, SIX6, SIX7 and SIX8 amplicons with 15 different pathogenic isolates of *Fusarium oxysporum* (1:15) and non-pathogenic isolate (Fo162).

Virulence potential	Isolate No.	Amplicons of SIX1	Amplicons of SIX5	Amplicons of SIX6
Hyper-virulent isolates	2	+	+	+
	4	+	+	+
	5	+	+	+
	15	-	+	+
Moderate isolates	1	+	-	-
	3	+	+	+
	6	+	-	-
	7	+	+	-
	9	+	-	+
	10	+	-	+
	11	+	-	-
	13	+	-	+
Hypo-virulent isolates	8	-	-	-
	12	-	-	-
	14	-	-	+

Table 4: Aggressiveness levels of 15 *Fusarium oxysporum* isolates and amplicons of SIX1, SIX5 and SIX6

the presence of SIX1, SIX5 and SIX6 genes within Egyptian isolates of *Fusarium oxysporum f. sp. lycopersici* can be used, for some extent, as a remarkable indicator for virulence potential on tomato plants under certain conditions. On the other hand, finding new tomato genotypes which contain immune genes against virulence effector genes Six1, SIX5 and SIX6 is highly recommended for using them in integrated pest management strategies in Egypt [24]. In deed, further investigations using many different pathogenic and non-pathogenic isolates belonging to various Fusarium forma speciales and representing different populations are needed to clarify the role of Fusarium virulence effector genes in pathogenicity process on different host plants.

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