Research Article

Horticultural Biotechnology Research 2016, 2: 1-7
http://scienceflora.org/journals/index.php/hbr/
doi: 10.19071/jhcbt.2016.v2.2996

Genetic variability among *Fusarium oxysporum* isolates from melon (*Cucumis melo*) in Qazvin province, Iran

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Received: 28.12.2015
Revised: 08.02.2016
Accepted: 03.03.2016
Published: 25.04.2016

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ABSTRACT

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *melonis* (Fom), the soilborne fungus that can due to more losses in yield of melon plants. *Fusarium* is a variable fungus in terms of morphology, symptom production, pathogenicity, and wide host range. From 2013 to 2015, 120 isolates of *F. oxysporum, Fusarium acuminatum, Fusarium graminearum, Fusarium proliferatum,* and *Fusarium solani* from melon were collected from disease suspected plants from the field and defined for pathogenicity in our melon plants. Then, a sort of those was elected for phylogenetic analysis. In the beginning, the *Fusarium* isolates were classified based on morphology and identifications were authenticated based on sequence data from elongation factor 1α and DNA and then were used to determine whether *Fusarium* variability is relevant to geographic origin and pathogenicity. Neighbor-joining analyses datasets indicated some clades based on geographic origin, but there is not even a single clades including solely of pathogens. Because of the factors affecting in pathogenicity are variable, we should be considered them in future studies. Due to the presence of Fom and some nonpathogenic isolates in almost all clades, it is obvious that Fom is not monophyletic.

KEY WORDS: *Fusarium* wilt, neighbor-joining, pathogenicity, phylogenetic

INTRODUCTION

Fusarium wilt is a soilborne disease which is the result of *Fusarium oxysporum* Schl. This fungi is one of the most important pathogens, which is causes wilting in some crops such as cantaloupe, cucumber, wax gourd, muskmelon, watermelon, and other types of melon. Melon (*Cucumis melo*) of the Cucurbitaceae family is one of the most important agricultural products in Qazvin Province, Iran. *Fusarium* is a soilborne fungus that is found in all soils and has global distribution. All isolates are able to survive in soil and growth in the rhizosphere area of many plant species. In the previous reports, more than 120 different formae speciales of *F. oxysporum* have been described based on host specificity (Armstrong and Armstrong, 1981). Wilting symptoms are the result of fungal spores and mycelium that block the xylem. Infected plants looking healthy at the beginning, but the vascular tissue is brown and discolored. To support morphological identification of *Fusarium* species, we can use of diversity in DNA sequences of genes. Furthermore, to estimate the genetic relationship for *F. oxysporum* we can use of phylogenetic analysis on DNA sequences (Hsuan *et al.*, 2011; Harrow *et al.*, 2010). Genetic diversity of a particular "forma specialis" revealed by using of multitude ways. Those include restriction fragment length polymorphism (Koenig *et al.*, 1997), random amplified polymorphic DNA (RAPD) (Windels, 1992), amplified fragment length polymorphism)Leslie *et al.*, 2006). There aren't any affective fungicides for controlling this pathogen on melon. Furthermore, crop rotation is not effective because isolates on the melon plant can live on the other crops (Gordon *et al.*, 1989). Hence, host resistance is the best way for disease management. To host plant resistance breeding, we must know if exists the variability within the pathogen population. Therefore, the present research study was performed to explain variation among *F. oxysporum* isolated from melon using DNA sequencing of translation elongation factor 1α (TEF1α). The goal of this study was to determine phylogenetic relationship among *F. oxysporum* isolates, gathered from melon plants, and determine diversity correlates with location origin and pathogenicity.

MATERIALS AND METHODS

In recent years, prevalence a disease had caused a severe decreasing in melon yield in Qazvin Province, Iran (Middle of Iran, 36°15'N - 50°0'E). Disease symptoms in melon farmlands were similar to *Fusarium* wilt. *F. oxysporum* consists of pathogenic and non-pathogenic strains that are morphologically indistinguishable.

Sampling, Identification, and Storage

Melon plants symptoms of *Fusarium* wilt were elected from melon farmland of Qazvin province from 2013 to 2015. Each sample was rinsed under running water for 20 min. Six pieces from root, crown, and stem (5 mm) were surface sterilized with 2% $NaCl_2$ for 2 min. These pieces were cultured onto potato dextrose agar (PDA; 200 g potato, 17 g glucose and 17 g agar/L) supplemented with 1 drop lactic acid. Petri dishes were incubated at 25°C for 10 days under fluorescent light (12 h photoperiod) (Nirenberg, 1976). Morphological identification was done on PDA and carnation leaf agar medium according to the morphological criteria keys of Nelson *et al.* (1983). For long-time storage, mycelium from *F. oxysporum* was stored desiccated on sterile glass fiber filter paper at −18°C (Hanson and Hill, 2004).

Pathogenicity Tests

A total of 120 *Fusarium* isolates cultured from diseased melon, tomato and watermelon were surveyed for pathogenicity on a *Fusarium* wilt susceptible melon plant Minoo 095P ergon (Ergon International N.V. The Netherlands). All isolates of *Fusarium* were grown in 250 ml volume conical flask, containing 100 ml Armstrong broth which is used for mass multiplication of inoculum of *Fusarium* (Booth, 1971). The flasks were incubated at 27°C, 120 rpm for 6 days into an incubator shaker. After 6 days, all cultures of *Fusarium* isolates were filtered through a muslin cloth before adjusting the spore density with distilled water to 2×10^7 spores/g of soil mixture in pots. Melon seedlings variety Minoo 095P was raised in steam sterilized soil. 21 days seedlings were transferred into the root trainers. The experiment was carried out in a greenhouse at 27°C and 13 h light period (600 lux). Plants were irrigated as and when required and supplied with Agrimel's solution weekly. Four replications were maintained for every isolate of *F. oxysporum*. The disease incidence was measured after 4 weeks of transplantation (Joshi *et al.*, 2013). All tests were conducted at least twice. Disease severity was recorded using a scale containing 4 grades suggested by Matsumoto *et al.* (2011): Grade: 0 = No symptoms, 1 = Small lesions on leaves, 2 = Leaves strongly affected, 3 = Plant death. After 4 weeks, roots and crown were removed to reisolate and confirmed the presence of *Fusarium* species. Disease index (DI) values were analyzed using PROC MIXED (SAS) with *t*-test (Dunnett, 1955), to determine if there were significant differences among our isolates or not. Half (50%) of the isolates from different locations and pathogenicity were selected for phylogenetic analysis (Hill *et al.*, 2011) (Table 1).

DNA Extraction from Fungal Cultures

Each fungal culture was inoculated in liquid potato dextrose broth and grown on a rotary shaker at 24°C for 5 days 100 rpm with 10 h of light per day and aeration. The fungal mycelium was harvested, ground to fine powder in liquid nitrogen. Genomic DNA was extracted from each culture by cetyltrimethylammonium bromide method. DNA samples were dried under vacuum and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), followed by incubation with RNase A at a concentration of 100 µg/mL at 37°C for 1 h (Gurjar *et al.,* 2009).

DNA Amplification

We used an intron region of TEF1α, to describe in *F. oxysporum* isolated from diseased melon plants. The primer used was described initially by O'Donnell *et al.* (1998) for EF1α. Amplification reactions were performed in volumes of 25 µl containing 1.5 µl of genomic DNA (25 mg), 1.5 µl of ×10 buffer polymerase chain reaction (PCR) (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8), 1 µl of Mg Cl2 (50 mM), 0.25 µl of dNTPs (100 mM), 0.2 µl of Taq DNA polymerase (5 U/ml), and 25 µl of each primer (20 mM). All reactions in our study were repeated at least twice.

Sequencing and Phylogenetic Analysis

The PCR products were purified using a Gene Elute PCR clean-up kit (Sigma, USA) pursuant to the manufacturer's protocol. The purified PCR products sent for sequencing to a service provider. The EF1α sequences were aligned with clustalx-2.1 (Thompson *et al.*, 1997). Then, default gap penalties were used in the pair-wise alignment. A neighbor-joining tree was delineated with the Jukes-Cantor model. The reliability of neighbor-joining trees was calculated by bootstrap method with 1000 random stepwise along with replicates. An isolate of *Fusarium culmorum* was selected as the outgroup.

RESULTS

Species Identification

Based on the morphological characteristics of isolates from farmlands, we identified *F. oxysporum, Fusarium acuminatum,*

Table 1: Geographic origin and pathogenicity on melon seedling (variety Minoo 095P) of *Fusarium oxysporum, Fusarium acuminatum, Fusarium graminearum, Fusarium proliferatum* and *Fusarium solani* cultured from melon, tomato and watermelon

Isolatea	Location	Morphological identification	Host	Year of isolation	Pathogenicity
F14	Takestan	*Oxysporum*	Melon	2013	NP
F16	Takestan	*Oxysporum*	Melon	2013	NP
F22	Takestan	*Oxysporum*	Melon	2015	NP
F23	Takestan	*Oxysporum*	Melon	2015	NP
R12	Buein zahra	*Oxysporum*	Melon	2013	P
R15	Buein zahra	*Oxysporum*	Melon	2013	P
R16	Buein zahra	*Oxysporum*	Melon	2013	P
R18	Buein zahra	*Oxysporum*	Melon	2013	P
R20	Buein zahra	*Oxysporum*	Melon	2015	P
R22	Buein zahra	*Oxysporum*	Melon	2015	P
R27	Buein zahra	*Oxysporum*	Melon	2015	LP
R30	Buein zahra	*Acuminatum*	Melon	2015	LP
R32	Buein zahra	*Acuminatum*	Melon	2015	LP
K6	Choobindar	*Oxysporum*	Melon	2013	P
K7	Choobindar	*Oxysporum*	Melon	2013	P
K8	Choobindar	*Oxysporum*	Melon	2013	P
K11	Choobindar	*Oxysporum*	Melon	2013	P
K12	Choobindar	*Oxysporum*	Melon	2015	LP
K15	Choobindar	*Solani*	Melon	2015	NP
S1	Sagez abad	*Oxysporum*	Melon	2013	P
S2	Sagez abad	*Oxysporum*	Melon	2013	P
S4	Sagez abad	*Oxysporum*	Watermelon	2014	NP
S6	Sagez abad	*Oxysporum*	Watermelon	2014	NP
S9	Sagez abad	*Oxysporum*	Watermelon	2015	NP
S10	Sagez abad	*Acuminatum*	Melon	2015	LP
D14	Danesfahan	*Oxysporum*	Melon	2013	P
D15	Danesfahan	*Oxysporum*	Tomato	2013	P
D19	Danesfahan	*Oxysporum*	Tomato	2013	LP
D29	Danesfahan	*Oxysporum*	Tomato	2014	P
D33	Danesfahan	*Oxysporum*	Tomato	2014	P
D37	Danesfahan	*Solani*	Melon	2015	NP
D41	Danesfahan	*Graminearum*	Melon	2015	NP
A10	Abyek	*Oxysporum*	Melon	2013	P
A11	Abyek	*Oxysporum*	Melon	2013	P
A12	Abyek	*Oxysporum*	Melon	2013	P
A15	Abyek	*Oxysporum*	Melon	2014	LP
A16	Abyek	*Oxysporum*	Watermelon	2014	NP
A17	Abyek	*Oxysporum*	Watermelon	2015	NP
A20	Abyek	*Proliferatum*	Melon	2015	NP
Q5	Qazvin	*Oxysporum*	Tomato	2013	P
Q6	Qazvin	*Oxysporum*	Tomato	2013	P
Q8	Qazvin	*Oxysporum*	Tomato	2013	NP
Q10	Qazvin	*Oxysporum*	Tomato	2013	P
Q13	Qazvin	*Oxysporum*	Melon	2014	P
Q19	Qazvin	*Solani*	Melon	2014	NP
Q20	Qazvin	*Proliferatum*	Melon	2014	NP
Q22	Qazvin	*Graminearum*	Melon	2015	NP
Q24	Qazvin	*Graminearum*	Melon	2015	NP
AL3	Alvand	*Oxysporum*	Melon	2013	P
AL4	Alvand	*Oxysporum*	Melon	2013	P
AL10	Alvand	*Oxysporum*	Melon	2014	LP
AL12	Alvand	*Oxysporum*	Melon	2014	P
AL18	Alvand	*Culmorum*	Melon	2015	NP
AL20	Alvand	*Proliferatum*	Melon	2015	NP
AV1	Avaj	*Oxysporum*	Melon	2013	P
AV2	Avaj	*Oxysporum*	Melon	2013	P
AV5	Avaj	*Oxysporum*	Melon	2014	P
AV6	Avaj	*Oxysporum*	Melon	2014	P
AV7	Avaj	*Oxysporum*	Watermelon	2014	NP
AV9	Avaj	*Oxysporum*	Watermelon	2015	NP

Those isolates that were significantly different from the water control at $P=0.05$ (Dunnett's one-tailed *t*-test) in all experiments are considered P; isolates not significantly different from controls are NP; and isolates were considered to have LP if it was significantly different than the control in some experiments but not others. P: Pathogenic, LP: Low pathogenicity, NP: Nonpathogenic

Fusarium graminearum, Fusarium proliferatum, and *Fusarium solani*. Identification based on morphological characteristics of our isolates was corroborated by comparison with sequences of their isolates in the GenBank.

Pathogenicity Testing and Determination

In the field and greenhouse, symptoms were include combinations of wilting, yellowing and chlorosis. Severely infected plants were wilted and died, but plants affected to a lesser degree became unproductive. In some cases, signs of decay on roots were observed. The most prominent internal symptom was vascular browning in plants. Symptoms of *Fusarium* wilt occurred after 37 days from the start. Reisolation was done from all the plants. Any signs of disease observed in the control plants.

All isolates were considered nonpathogenic (NP) in the experiment, when the *F*-test indicated no significant differences among our isolates and also control (Table 1). When our isolates indicated significant differences, *t*-test was used to compare all isolates with the control treatment, with $P = 0.05$. Those isolates (at least two times of experiments) which were significantly higher DI than the control treatment were calculated pathogenic (P). Those isolates which had a significant differences from the control treatment in few experiments but not all of the experiments, were calculated, low pathogenic (LP). 26 were identified as *F. oxysporum* and surveyed for pathogenicity on melon seedlings among of 50 *Fusarium* species that were gathered from melon and tomato in 2013. All of these were pathogenic in pathogenicity test on melon seedlings. In 2014, 40 *Fusarium* cultures were isolated from melon that 12 were identified as *F. oxysporum* and surveyed for pathogenicity. Six isolates of these isolates were pathogenic on melon plants. Furthermore, one isolate of *F. solani* and *F. proliferatum* identified that were NP. In 2015, 30 *Fusarium* cultures were isolated from melon that 9 isolates of *F. oxysporum* were collected from melon and watermelon were tested, and of these isolates, only three were pathogenic on melon plants. Seven *F. oxysporum*

cultures isolated from watermelon in 2014 and 2015 (S4, S6, S9, A16, A17, AV7, AV9) were distinguished as non-pathogenic on melon seedlings. Moreover, three *F. acuminatum* were identified that all of them were LP. We had three *F. graminearum* cultures isolates from melon that all of them were non-pathogenic on melon seedlings (Table 1).

Phylogenetic Analysis

Phylogenetic tree from neighbor-joining showed progressively increasing resolution. Datasets were including five small groups of isolates (III-VIII) and two large groups (I, II) (Figure 1). Group (I) is resolved into smaller groups and groups (V-VIII) were found at the base of the tree (Figure 1). In clade (I), *F. oxysporum* from melon and watermelon grouped with NP (F14, F16, F22, F23, S4, S6, S9, AV7, AV9) from melon.

Moreover, 12 pathogenic *F. oxysporum* isolates from melon dissolved in clade (I) with a support value of 94%; and one of LP *F. oxysporum* isolate (R27) situated with the pathogenic *F. oxysporum* isolates with 95% support. Clade (II) contained pathogenic *F. oxysporum* from Choobindar (K6, K7, K8, and K11), Danesfahan (D14, D15, D29, and D33) and Alvand (AL3, AL4, and AL12); LP *F. oxysporum* from Choobindar (K12), Danesfahan (D19) and Alvand (AL10). Group (III) (Qazvin *F. oxysporum* isolates Q5, Q6, Q10, and Q13) situated outside of two large groups (I and II). They were

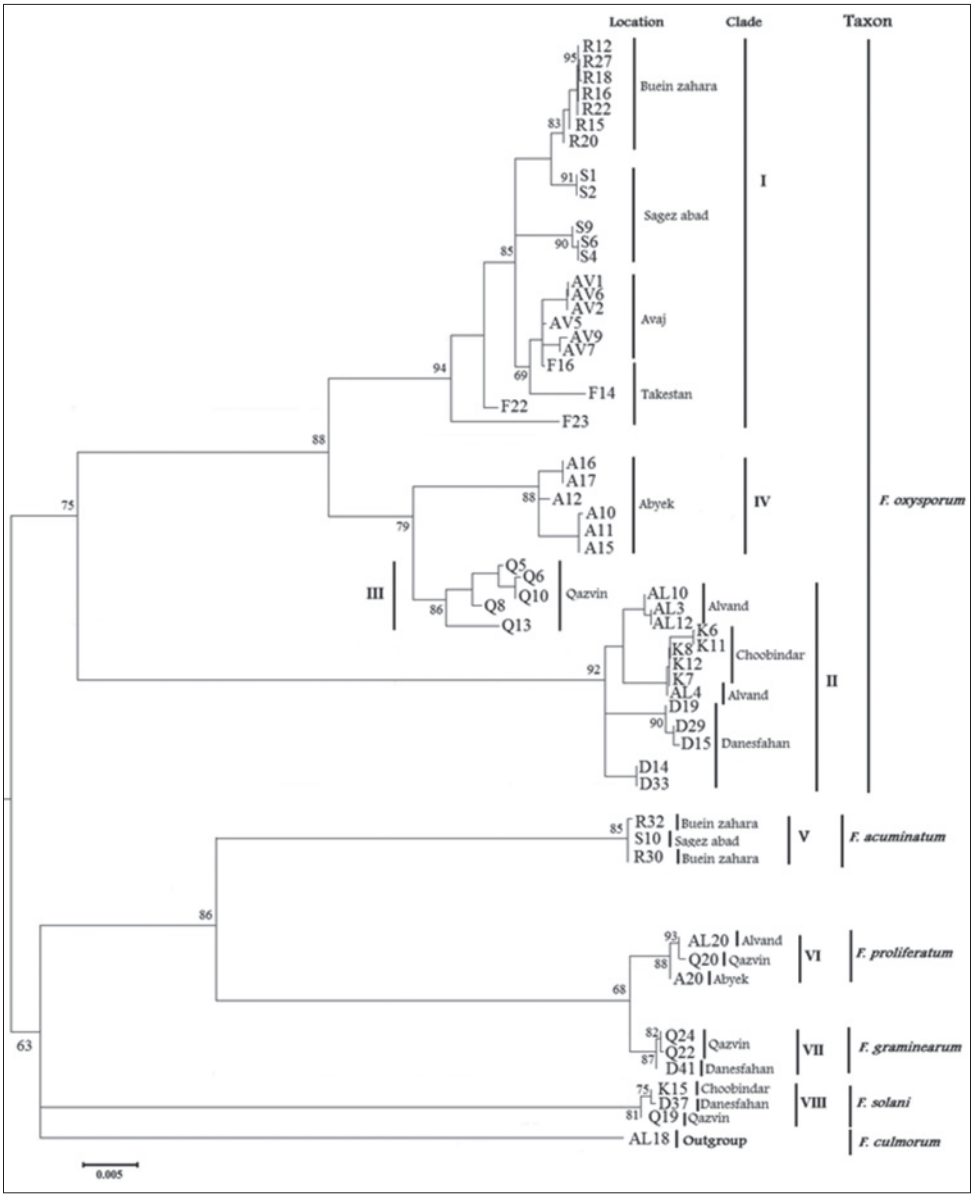

Figure 1: Neighbor-joining tree of 60 isolates of *Fusarium oxysporum* inferred from translation elongation factor 1α using Jukes-Cantor method. An isolate of *F. culmorum* was used as the out group

all pathogenic on melon seedlings except Q8 that was NP. Clade (IV) contained pathogenic *F. oxysporum* from Abyek (A10, A11 and A12); LP *F. oxysporum* from Abyek (A15), and NP *F. oxysporum* from Abyek (A16 and A17 from watermelon plants).

Groups (V) (Buein zahra and Sagez abad *F. acuminatum* isolates R30, R32, and S10), (VI) (*F. proliferatum* isolates A20, Q20, and AL20), (VII) (Qazvin and Danesfahan *F. graminearum* isolates D41, Q22, and Q24) and (VIII) (*F. solani* isolates K15, D37, and Q19) also situated outside of two large groups (I and II) and in analyses were separated phylogenetically from isolates of *F. oxysporum*.

DISCUSSION

F. oxysporum is due to vascular wilt diseases in a large variety of important crops (Beckman, 1987). *Fusarium* can attack to vascular system of the plants and secrete its toxins, which cause the vascular wilting and then death within a few days or weeks (Champaco *et al.*, 1993). Phylogenetic relationships in *F. oxysporum* have been studied by many researchers to determine evolutionary orientation among *Fusarium* species and to distinguish the dynamism in the different formae speciales (Gordon and Martyn, 1997). Using conserved gene regions in some of studies have showed that formae speciales of *F. oxysporum* are monophyletic, but some of other studies have distinguished polyphyletic groups (Koenig *et al.*, 1997; Jimenez Gasco *et al.*, 2002).

In this study, the phylogeny and the pathogenicity of *F. oxysporum* isolates were analyzed. The results indicated the genetic variability in *Fusarium* spp. from different locations in Qazvin Province. They indicated the genetic similarities and differences among the tested *Fusarium* spp. In this study, *F. oxysporum* strains from melon being placed in clades next and close to *F. oxysporum* strains from watermelon and tomato that show probably *F. oxysporum* from melon plants is polyphyletic. Clade (I) contained of pathogenic and NP isolates from Takestan, Buein zahra, Sagez abad, and Avaj. Phylogenetic analyses of EF1α sequences showed isolates in four major groups and four minor groups that all four major groups were *F. oxysporum* sequences from melon, tomato, and watermelon.

Neighbor-joining analysis of EF1α with the highest support values demonstrate in the most clades (more than 70). The pathogenic and LP isolates are in clade (II) including isolates from Alvand, Choobindar, and Danesfahan. The pathogenic isolates are in clade (III) including isolates from Qazvin. The pathogenic, LP and NP isolates are in clade (IV) consisted isolates from Abyek. The isolates of *F. solani, F. proliferatum, F. acuminatum,* and *F. graminearum* evaluated form four clades separated each other which situate out of the four major clades. The identification isolates of *F. oxysporum, F. solani, F. proliferatum, F. acuminatum,* and *F. graminearum* by morphological methods supported with the results of the sequence of BLAST analysis.

The phylogenetic analyses indicated *F. oxysporum* from watermelon and tomato clustered with *F. oxysporum* from melon in most of clades. Furthermore, proofs for cross pathogenicity were recorded in pathogenicity tests where seven isolates of *F. oxysporum* from tomato used in this research were pathogenic on melon seedlings variety Minoo 095P. Many investigators have been reported pathogenic populations in the fungi (Koenig *et al.*, 1997). However, genetic resistance may enable management in some locations, but some of the farmers have announced that managing of *Fusarium* wilt is decreased in different conditions. Although, they are ubiquitous but, the variability and pathogenicity of these isolates are rarely reported (Gordon and Okamoto, 1992).

A lot of studies of genetic diversity have been presented the possible correlation between genotypes and pathotypes (Nelson *et al.*, 1997; Vakalounaki and Fragkiadakis, 1999). Variability in resistance in different locations shows races within *F. oxysporum* f. sp. *melonis* (Fom) (Jimenez-Gasco *et al.*, 2002). It can propose races in Fom has been firmly-fixed on cross-pathogenicity among of isolates of melon, tomato and watermelon. However, in this study genetic variability and geographical locations had no obvious correlation for the *F. oxysporum* isolates. This is similar to a lack of correlation between the UP-PCR (by EF1α) and geographic location that is reported for *F. oxysporum* (Zhao *et al.*, 2014). This is the opposite of a correlation observed between the RAPD pattern and geographic locations in a previous research study (Assigbetse *et al.*, 1994).

Both in the field and greenhouse, the environmental conditions may participate diversity in pathogenicity response. Many of researchers found that inoculation quantity, plant variety, inoculation method, plant stage, and temperature can effect on the pathogenicity of Fom isolates. The cross pathogenicity of *F. oxysporum* isolates and their polyphyletic nature can be get the resistance breeding attempts much tough than other fungi. These findings would be required to assess tactics of resistance breeding in field or greenhouse conditions.

This research study indicates the first molecular phylogenetic studies of *F. oxysporum* from melon in Qazvin province, Iran as compared to Zhao *et al*. (2014), which compared some species using EF1α in Liaoning Province, China. This work supports the importance of expanding genetic markers to achieve a better perception of the evolutionary structure of fungi especially Fom. Furthermore, This study was include more geographical locations and similar numbers of isolates from each geographical location, so showed a more precise picture of aggregations between location origin, pathogenicity and phylogeny than other research studies.

ACKNOWLEDGMENTS

The author would like to thank the Agricultural Organization of Qazvin Province. This work is done with Personal expenses and without any help from Bu-Ali Sina University.

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