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Research Article

Morphological and molecular identification of *Fusarium* spp. associated with carnation *Dianthus caryophyllus* in Mahallat, Iran

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Abstract: Carnation is an ornamental plant of economic importance worldwide. *Fusarium* fungus is one of the critical infectious agents of Carnation in Mahallat city. Some species of *Fusarium* cause wilting and crown and root rot in carnation. In this study, 20 fungal isolates of *Fusarium* were isolated from diseased carnation plants and rhizosphere samples from Carnation greenhouses in Mahallat. The isolates were identified morphologically using Leslie and Summerell's identification key. Amplification of the *TEF-1α* gene region was done using ef1 and ef2 primers. Amplified fragments were purified and sequenced, and the nucleotide sequences were introduced in the *Fusarium* ID database for molecular identification of isolates based on the *TEF-1α* gene. A phylogenetic tree was also drawn. The results showed that *Fusarium* isolates were categorized into five species: F14 was identified as *Fusarium brachygibbosum* morphologically and *F. solani* molecularly. F6 was morphologically and molecularly identified as *F. solani,* F10 identified as *Fusarium culmorum*. All crown and root isolates (F11, F12, F13, F15, F16, F17, F18, F9, and F20) were identified as *F. oxysporum.* The rhizosphere isolates of F1, F2, F3, F4, F5, F7, F8, and F9 were identified as *Fusarium proliferatum.* This study shows that the use of the *TEF-1α* gene for molecular identification of *Fusarium* isolates is a relatively accurate, fast, and straightforward method for detecting species and can be used in plant pathological studies. Identifying the pathogen is the first step to controlling the disease.

Keywords: Carnation, *Fusarium*, molecular identification**,** *TEF-1α* gene

Introduction

Mahallat city in Markazi province is an important center for producing flowers and ornamental plants in Iran. Carnation *Dianthus caryophyllus* L. belongs to the family Caryophyllaceae and is one of Iran's four central cut flowers for export. The genus Dianthus includes more than 300 species, several of which are sold as ornamental garden plants, pots, or cut branches. *Fusarium* species are economically important as pathogens on most crops in the world. Also, some of the *Fusarium* species are known as saprophytes. Diseases caused by *Fusarium* spp. in carnations include wilting, root, and crown rot that cause damage to carnation greenhouses every year. *Fusarium* wilt is one of the most destructive diseases in most carnation-growing regions of the world (Kermajany *et al.*, 2017). This disease was first reported in Iran in 1996 by Varamin (Etebarian, 1996). Fattahi *et al.* (2013) identified the *F.oxysporum*, *F. proliferatum*, *F.*

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solani, and *F. equiseti* in carnation greenhouses in Iran and reported wilt for the first time disease caused by *F. proliferatum* and *F. solani* on carnation in Iran.

Accurate identification of *Fusarium* species in carnation greenhouses is the first step in combating diseases caused by this pathogenic fungus. Morphological specification of fungi is based on the size, shape, absence, or presence of asexual reproduction structures in different culture media (Leslie and Summerell, 2006). Molecular identification can be made using DNA-based procedures, such as conserved sequencing regions. The TEF-1α gene encoding translation elongation factor 1α (TEF-1α) is suitable in sequence analysis for taxonomic and phylogenetic studies (Geiser *et al.*, 2004). It appears that this gene in *Fusarium* is consistently single-copy. It shows a high level of sequence polymorphism among closely related species. That is why TEF-1α is a marker of choice as a single-locus recognition tool in *Fusarium* (Geiser *et al.*, 2004).

This study aimed to identify the morphological and molecular characteristics of Fusarium species associated with carnation in Mahallat. According to our study, molecular identification of carnation fusarium has not been performed in Iran so far. So, carnation plants with disease and infected rhizosphere symptoms were collected from infected greenhouses in Mahallat city in Markazi province. After culturing the samples and purifying the fungus, morphological identification was performed using the Leslie and Summerell identification key (Leslie and Summerell, 2006). Molecular identification was also based on the TEF-1α gene. (Geiser *et al.*, 2004).

Materials and Methods

Sample collection and isolation of *Fusarium* species

The *Fusarium* Isolates were isolated from the diseased carnation plants and their rhizosphere in Mahalat during the growing season in 2019. From among 20 *Fusarium* isolates obtained from Carnation grown in greenhouses, isolates F1 to F10 were obtained from the rhizosphere, and isolated F11 to F20 were isolated from the crown and roots of infected plants.

The infected plant tissues were surface sterilized with ethanol 80 % and transferred onto potato dextrose agar medium (Peeled potato; 250 g, Dextrose; 20 g, Distilled water1000 mL at pH 7.0) in Petri dishes. The Petri dishes were incubated at room temperature (28 ± 2 °C) observed periodically to grow colonies. The pure colonies were obtained by the single spore method. The pure colonies were transferred to the PDA Petri plate to maintain the culture.

Morphological identification

The Carnation leaf culture medium (CLA and KCL-agar) was used to identify *Fusarium* species. Morphological identification of isolates was made based on the morphological characteristics of macroconidia, the presence or absence of microconidia. If so, their shape and position at the end of conidiophore (single, chain, and false heads), type of phialides (monophialide or polyphialide), presence or absence of chlamydospores, as well as colony characteristics were determined on potato dextrose agar (PDA) medium, including mycelium pigmentation and rate of colony growth using Leslie and Summerell identification key (Leslie and Summerell, 2006). The pathogenicity test was performed according to the method described by Moeini *et al*. (2014).

Molecular identification of *Fusarium* species

DNA extraction was performed from fungal mycelium using the extraction kit and instructions of Pars Toos Company. DNA concentration was estimated using a UV–vis spectrophotometer (Thermo Spectronic UV1). It was then stored at 20 °C. A standard polymerase chain reaction (PCR) was used to amplify the TEF-1α gene region using ef1 (5-ATGGGTAAGGA(A/G)GACAAGAC-3) as the forward primer and ef2 (5-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3)

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as a reverse primer (Geiser *et al*., 2004). These primers amplify a 700 bp region of TEF-1α in all known fusaria.

The PCR was performed in ThermoCycler (BIORAD® T100TM, Germany) as the following program: Initial denaturation at 94 °C for 5 min followed by 30 cycles at 94 °C for 1 min, 55 °C for 50 s, and 72 °C for 3 min and a final extension at 72 C for 10 min. Amplified PCR products were separated on an agarose gel (1.5% w/v) in 1X TAE buffer at 100 V. The 100bp DNA marker (MBI Fermentas, Lithuania) was used to estimate DNA band size. Negative control was used to test the absence of contamination.

DNA Sequencing

Isolation of 700 bp band of DNA was done under UV. Separated bands were purified and sequenced by the South Korean Macrogene Company (http://dna.macrogen.com, Korea). The sequences for TEF-1α areas were first reviewed with the BioEdit software, and then sequences were presented in the Gene Bank database (www.ncbi.nlm.nih.gov/BLAST). The sequences were registered on the GenBank of NCBI site (https://www.ncbi.nlm.nih.gov/WebSub/). The nucleotide sequences also were introduced in the *Fusarium* ID database (http://isolate.*Fusarium*db.org/blast.php) for molecular identification of isolates based on translation elongation factor 1 alpha (TEF-1α gene). Phylogenetic analysis was done using the ClustalW (https://www.genome.jp/tools-bin/clustalw) program.

Phylogenetic analysis

The newly obtained elongation factor 1-alpha (EF1-α) sequences of *Fusarium* spp. (from F1 to F20), together with the other sequences of the *Fusarium* genus already used by Laurence *et al*. (2011), Herron *et al*. (2015), Papizadeh *et al*. (2018), and Al-Hatmi *et al*. (2018) were selected for phylogenetic analyses. The dataset was updated by investigations in the database for acquiring correct sequences. The outgroup taxa for the present dataset were taken based on Hirooka *et al*. (2011). All sequences were aligned using the Q-INS-i algorithm of MAFFT version 7 (http://mafft.cbrc.jp/ alignment/ server/) (Katoh and Standley, 2013). The online version of Gblocks 0.91b (Castresana, 2000) was applied to remove ambiguous parts of the alignment, with all three options (including allow smaller final blocks, allow gap positions within the final blocks, and allow less strict flanking position) for a fewer stringent selection (http://molevol.cmima.csic.es/ castresana/Gblocks_server.html). The most suitable substitution model for the dataset was chosen using the Akaike information criterion (AIC) using PAUP* /MrModeltest v2.2 (Nylander, 2004). Asymmetrical model including a gamma distribution for rates (GTR + G) was picked out for EF1-α analysis. Bayesian inference (BI) was carried out using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003), choosing a random beginning tree and running the chains for 4 million for EF1-α. After casting off burn-in samples, the residual samples were reserved for additional analyses. The Markov Chain Monte Carlo (MCMC) method within a Bayesian framework was utilized to assess the posterior probabilities of the phylogenetic trees (Larget and Simon, 1999) by the 50% majority rule. The obtained phylogenetic tree was visualized via Dendroscope V.3.2.8 (Huson and Scornavacca, 2012).

Results

Morphological identification of *Fusarium* isolates

Fusarium isolates were identified morphologically. Based on structures of macroconidia, microconidia, and other morphological characters (Fig. 1), the isolates of F1, F2, F3, F4, F5, F7, F8, and F9 were identified as *F. proliferatum*. The isolates of F11, F12, F13, F15, F16, F17, F18, F9, and F20 were identified as *F. oxysporum*. Also, the isolates F6, F10, and F14 as *F. solani*, F. culmorum, and *F. brachygibbosum,* respectively. All *F. oxysporum* isolates and *F. brachygibbosum* were isolated from the roots

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and crowns of wilted plants. At the same time, *F. proliferatum* and *F. culmorum* were isolated from the rhizosphere. Moreover, molecular identification recognized the *F. brachygibbosum* as *F. oxysporum*. The vascular browning, root, and crown rot symptoms were visible in diseased plants in the pathogenicity test (Fig. 2).

Figs. 1 and 3 show the morphological characteristics of these isolates. Table 1 shows the results of morphological characterization of *Fusarium* spp. Isolates (Table 1).

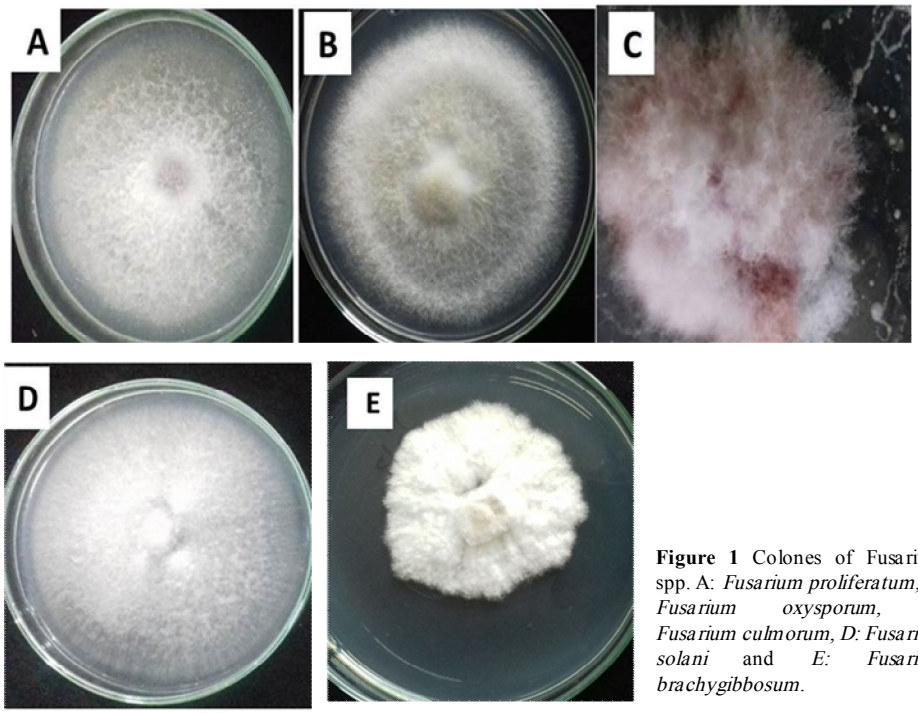

Figure 1 Colones of Fusarium spp. A: *Fusarium proliferatum, B: Fusarium oxysporum, C: Fusarium culmorum, D: Fusarium solani* and *E: Fusarium brachygibbosum*.

Figure 2 Necrosis and browning of the vessels in the crown region of diseased carnation in the greenhouse in pathogenicity test of *Fusarium* isolates A: F14, B: F13, C: F17, and D: F19.

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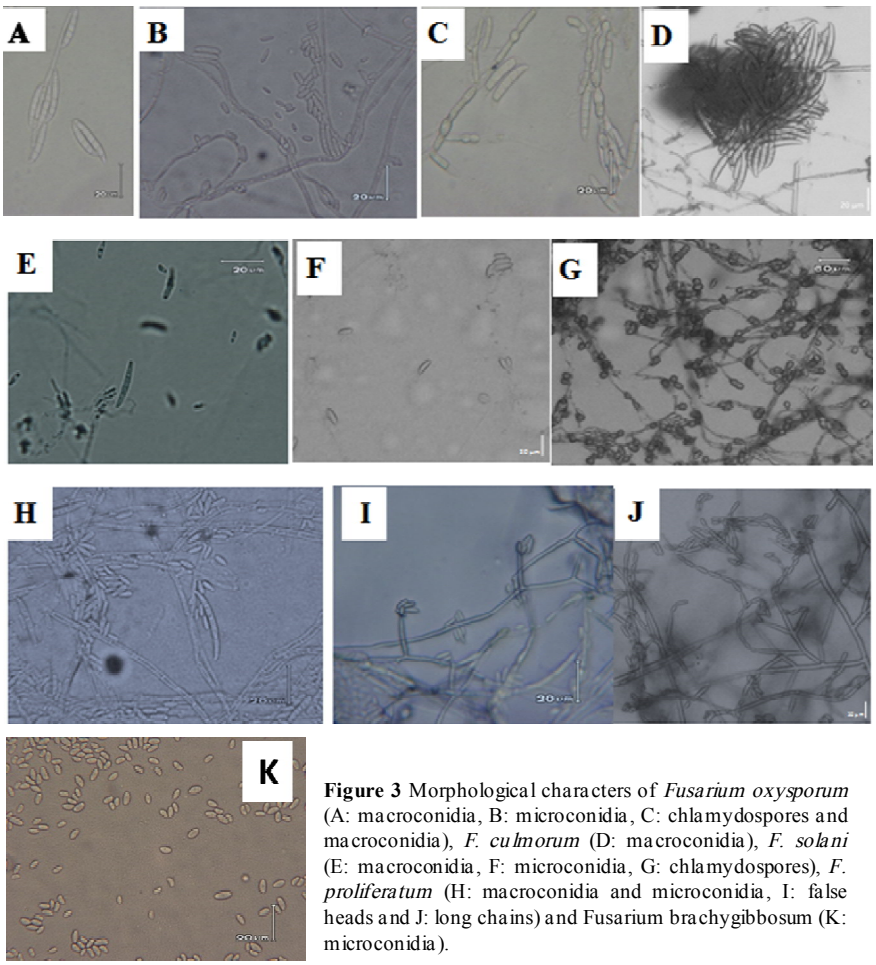

Figure 3 Morphological characters of *Fusarium oxysporum* (A: macroconidia, B: microconidia, C: chlamydospores and macroconidia), *F. culmorum* (D: macroconidia), *F. solani* (E: macroconidia, F: microconidia, G: chlamydospores), *F. proliferatum* (H: macroconidia and microconidia, I: false heads and J: long chains) and Fusarium brachygibbosum (K: microconidia).

Table 1 *Morphological characteristics of* Fusarium *spp. isolated from rhizosphere, crown, and root of* Dianthus caryophyllus.

Entries	*F. proliferatum*	*F. oxysporum*	*F. solani*	*F. culmorum*
Pigmentation on PDA	white but may become purple-violet with age	white to pale violet	White to cream	pale orange but becomes brown
Growth rate	R	R	R	R
Macroconidia	+	+	+	+
Apical cell	Cu	Ta and cu	Bl and Ro	Bl and Ro
Basal cell	Pd	Fs	Fs or pd	Nwfs
Septate	3-5	3	5-7	3-4
Size (µm)	27-90 × 3-6	40-85 × 3.5-9	30-72 × 5-8	30-60 × 5-8
Microconidia	+	+	+	-
Form	Lc, Fh	Fh	Fh	-
Phialides	Mono, Poly	Mono	Mono	-
Shape	Cl, Py	Ov, Ki	Ov, Re, Fu	-
Septate	0	0	0-2	-
Size (µm)	7-16.5 × 2-5	6.9-8.9 × 3-5	7-15 × 2-4	-
Clamydospore	-	+	+	+

+: Presence, -: Absence, Cu: Curved, Bl: Blunt, Ro: Rounded, Ta: Tapered, Pd: Poorly developed, Fs: Foot shape, Nwfs: Notched and without a distinct foot shape, Lc: Long chains, Fh: False heads, Cl: Club, Py: Pyriform, Ov: Oval, Re: Reniform, Fu: Fusiform, Ki: Kidney shaped.

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Molecular identification of *Fusarium* species

Primers ef1/ef2 successfully identified and amplified a 700bp fragment containing the *TEF-1α* gene in *Fusarium* species. The presence of a clear band in fungal isolates indicates the correct amplification of this gene. Also, no amplicon was recorded in the negative control (Fig. 4).

Molecular identification results based on the TEF-1α gene with average identities of 99.33% supported morphological identification results. The only exception was the F14 isolate, which was morphologically identified as *F. brachygibbosum* and molecularly as F. solani, using the *Fusarium* ID site with 93.87% identity. This isolate in the BLAST site with 93% identities was recognized as *F. metavorans*. *Fusarium* species were identified based on the translation-elongation factor -1 alpha gene (TEF-1α), according to table 2.

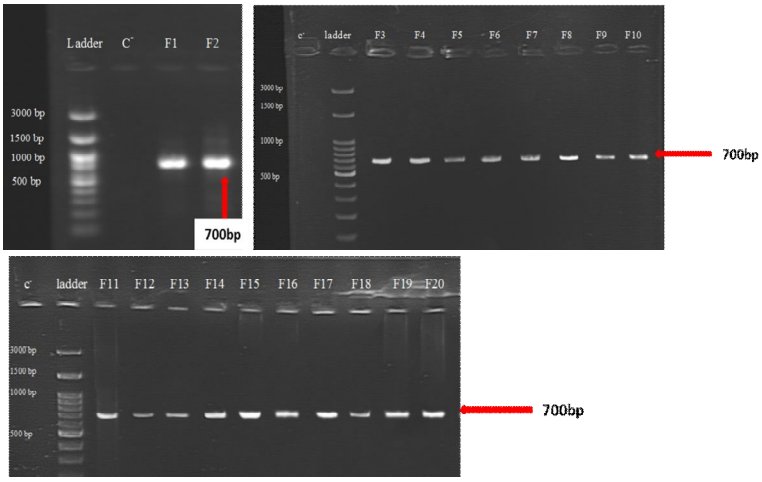

Figure 4 Amplification of conserved region of TEF-1α gene. C: 100 bp DNA ladder, F1-F20: fusarium isolates.

Table 2 Characterization of *Fusarium* isolates and their GenBank accession number.

Fungi ID	Host	Isolation source	Morphological identification	Molecular identification based on TEF-1A-1α (% similarity)	GenBank Accession number
F1	*D. caryophyllus*	Rhizosphere	*F. proliferatum*	*F. proliferatum* (99.07%)	MT723967
F2	*D. caryophyllus*	Rhizosphere	*F. proliferatum*	*F. proliferatum* (99.07%)	MT723968
F3	*D. caryophyllus*	Rhizosphere	*F. proliferatum*	*F. proliferatum* (99.69%)	MT723969
F4	*D. caryophyllus*	Rhizosphere	*F. proliferatum*	*F. proliferatum* (99.24%)	MT723975
F5	*D. caryophyllus*	Rhizosphere	*F. proliferatum*	*F. proliferatum* (97.92%)	MT723977
F6	*D. caryophyllus*	Rhizosphere	*F. solani*	*F. solani* (98.78%)	MT723976
F7	*D. caryophyllus*	Rhizosphere	*F. proliferatum*	*F. proliferatum* (98.74%)	MT723978
F8	*D. caryophyllus*	Rhizosphere	*F. proliferatum*	*F. proliferatum* (99.4%)	MT723979
F9	*D. caryophyllus*	Rhizosphere	*F. proliferatum*	*F. proliferatum* (97.53%)	MT723980
F10	*D. caryophyllus*	Rhizosphere	*F. culmorum*	*F. culmorum* (98.87%)	MT753012
F11	*D. caryophyllus*	Crown and root	*F. oxysporum*	*F. oxysporum* (98.58%)	MT753011
F12	*D. caryophyllus*	Crown and root	*F. oxysporum*	*F. oxysporum* (99.84%)	MT723981
F13	*D. caryophyllus*	Crown and root	*F. oxysporum*	*F. oxysporum* (100%)	MT723971
F14	*D. caryophyllus*	Crown and root	*F. brachygibbosum*	*F. solani* (93.87%)	MT681918
F15	*D. caryophyllus*	Crown and root	*F. oxysporum*	*F. oxysporum* (99.84%)	MT723970
F16	*D. caryophyllus*	Crown and root	*F. oxysporum*	*F. oxysporum* (99.84%)	MT723982
F17	*D. caryophyllus*	Crown and root	*F. oxysporum*	*F. oxysporum* (100%)	MT723972
F18	*D. caryophyllus*	Crown and root	*F. oxysporum*	*F. oxysporum* (99.85%)	MT723983
F19	*D. caryophyllus*	Crown and root	*F. oxysporum*	*F. oxysporum* (99.7%)	MT723973
F20	*D. caryophyllus*	Crown and root	*F. oxysporum*	*F. oxysporum* (100%)	MT723974

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The TEF-1α partial gene sequences of *Fusarium* isolates were analysed to conduct a phylogenetic tree. As shown in Figure 5, *Fusarium* isolates were divided into five general groups. The first group consisted only of F14 isolate that was identified *F. brachygibbosum* morphologically and *F. solani* molecularly. This isolate was far from the rest. The second group consisted of F6 that identified as *F. solani* morphologically and molecularly. The third group consisted of F10 identified as *F. culmorum.* The fourth group included F1, F2, F3, F4, F5, F7, F8, and F9, all of which were identified as *F. proliferatum.* The fifth group comprised F11, F12, F13, F15, F16, F17, F18, F9, and F20, identified as *F. oxysporum***.** Thus, phylogenetic analysis was able to differentiate *Fusarium* species based on the TEF-1α gene.

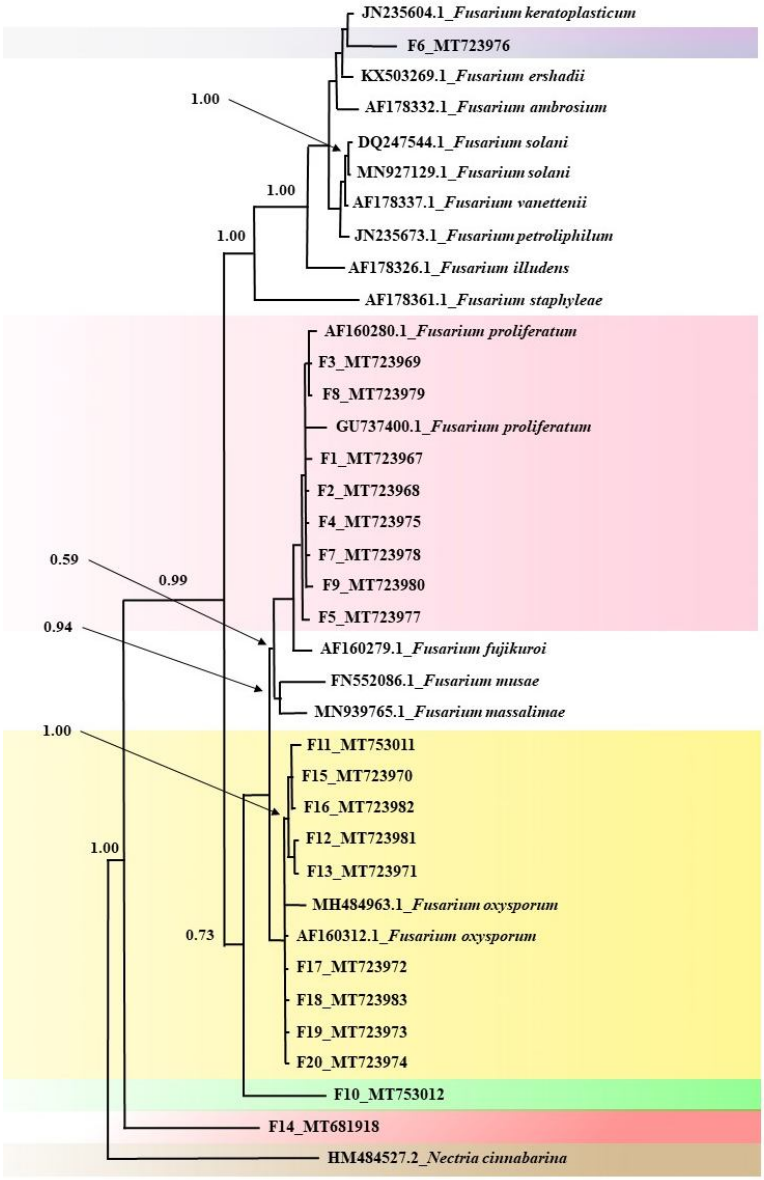

Figure 5 Bayesian 50% majority rule consensus tree deduced using EF1-α sequences of the *Fusarium* spp. under the GTR + G model. The newly generated sequences are in color frameworks.

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Discussion

Since *Fusarium* species carnation is among the most severe pathogens in greenhouses, accurate identification of carnation related *Fusarium* species can be the first step in combating *Fusarium* disease and preventing damage to this plant. The purpose of this study is the identification of *Fusarium* spp. associated with carnation. In this research, molecular identification of *Fusarium* isolates detected the four species, including *F. oxysporum*, *F. proliferatum*, *F. solani*, and *F. culmorum*, in the order of frequency. *F. oxysporum* is known as a wilting agent in carnation. *F. oxysporum* seems to be one of the most harmful pathogens for greenhouse crops in Iran. Also, Sahampoor *et al*. (2020) mentioned *F. oxysporum* f.sp. *radicis-cucumerinum* as an important pathogen in greenhouse cucumber. They considered the use of *Trichoderma* to be effective in laboratory control of this pathogenic fungus, and by using gamma rays, they increased the efficiency of *Trichoderma* in the control of *F. oxysporum*. Fattahi *et al.* (2013) also reported wilt disease caused by *F. proliferatum* and *F. solani* on carnation in Iran. Although there are many nonpathogenic species of *Fusarium*, all *F. oxysporum* species were isolated from diseased plants were pathogens based on the results of the pathogenicity test. Of the 20 *Fusarium* isolates collected in Mahalat, *F.oxysporum* was the most prevalent with 45% (9 of 20 isolates), followed by *F. proliferatum* with a frequency of 40% (8 of 20 isolates). The least frequent isolate was *F. culmorum,* with a frequency of 5% (1 of 20 isolates). The use of the TEF-1α gene, which encodes an essential part of the 1α translation elongation factor, has been suggested as a useful phylogenetic tool for detecting Fusarium species. This gene is highly informative at the species level in *Fusarium*, and non-orthologous copies of the gene have not been detected in the genus. The primers of ef1 and ef2 can successfully amplify this region for all Fusarium species (Geiser *et al.*, 2004).

Camacho López *et al.* identified *Fusarium oxysporum*, *F. proliferatum*, *F. globosum*, *F. solani*, *F.incarnatum, F. equiseti*, and *F. tricinctum* that were associated with diseased carnation plants in Baja California. Molecular identification of these isolates was performed based on the TEF-1α gene. (Camacho López *et al.*, 2014). Rezaee *et al*. (2018) identified races of *Fusarium solani* f. sp. cucurbitae using specific primers based on TEF-1α gene. Rabiei-Motlagh *et al*. (2017) showed that PCR assay using *Avr1*, *Avr2,* and *Avr3* genes could distinguish between nonpathogenic and low virulence strains of *Fusarium oxysporum* f. sp. *lycopersici*.

Considering the correlation between molecular identification and morphological results, it seems that the molecular method can be faster and more straightforward for identifying these pathogens. In this research, the TEF-1α gene was well able to identify and distinguish species. The results of this study can help identify diseases caused by *Fusarium* in carnation greenhouses.

Conflict of Interests

The authors declare that they have no conflicting interests.

Authors' Contributions

Samira Shahbazi has collected, isolated, and purified fungal isolates, contributed to writing the discussion (Research project collaborator); Fatemeh Zaker Tavallaie designed experiment, Performed molecular identification and gene registration in NCBI and wrote the manuscript (Executor of the research project) and Zoha daroodi performed morphological identification, DNA extraction and PCR amplification (Research project collaborator).

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شناسایی مورفولوژیکی و مولکولی فوزاریوم‌های مرتبط با میخک *Dianthus caryophyllus* در ایران، محلات

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چکیده: میخک یک گیاه زینتی با اهمیت اقتصادی در سراسر جهان است. قارچ *Fusarium* یکی از عوامل مهم آلودگی میخک در شهرستان محلات است. بعضی از گونه‌های *Fusarium* باعث پژمردگی و پوسیدگی طوقه و ریشه در میخک می‌شوند. در این مطالعه، ۲۰ جدایه قارچی از جنس *Fusarium* از گیاهان میخک دارای علائم بیماری و هم‌چنین ریزوسفر این گیاهان از گلخانه‌های گل میخک در محلات جداسازی شدند. جدایه‌ها از نظر مورفولوژی با استفاده از کلید شناسایی Leslie و Summerell شناسایی شدند. تکثیر ناحیه ژنی TEF-1α با استفاده از آغازگرهای ef1 و ef2 انجام شد. قطعات تکثیر شده جداسازی و توالی‌یابی شدند. این توالی‌های نوکلئوتیدی در پایگاه داده Fusarium ID جهت شناسایی مولکولی جدایه‌ها براساس ژن TEF-1α وارد شدند و درخت فیلوژنتیکی نیز رسم شد. نتایج نشان داد که جدایه‌های فوزاریوم به پنج گروه تقسیم می‌شوند: F14 از نظر ریخت‌شناسی به‌عنوان *Fusarium brachygibbosum* و از لحاظ مولکولی *F. solani* شناخته شد. F6 از نظر ریخت‌شناسی و مولکولی به‌عنوان *F. solani*، F10 به‌عنوان *F. culmorum* شناسایی شد. سایر جداشده‌های طوقه و ریشه (F11، F12، F13، F15، F16، F17، F18، F19 و F20) به‌عنوان *F. oxysporum* شناخته شدند. جدایه‌های ناحیه ریزوسفر (F1، F2، F3، F4، F5، F7، F8 و F9) به‌عنوان *F. proliferatum* تشخیص داده شدند. این مطالعه نشان داد که استفاده از ژن TEF-1α برای شناسایی مولکولی جدایه‌های *Fusarium* یک روش نسبتاً دقیق، سریع و ساده برای تشخیص گونه‌های قارچ است و می‌تواند در مطالعات آسیب‌شناسی گیاه مورد استفاده قرار گیرد. شناسایی پاتوژن اولین قدم برای کنترل بیماری است.

واژگان کلیدی: میخک، فوزاریوم، شناسایی مولکولی، ژن TEF-1α

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