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Morphological and Molecular Identification of Fusarium Associated with Beans in Selseleh County

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ABSTRACT

Fusarium species are among the important factors in bean contamination worldwide, some of which cause wilting and rotting of crowns and roots in beans. Identifying *Fusarium* species is necessary to prevent wilting and root and crown rot diseases in bean fields. In this study, 60 fungal isolates showing disease symptoms were collected from the bean samples. The rhizosphere, including the *Fusarium* species complex of *Fusarium solani*, *F. oxysporum*, *F. equiseti*, *F. acuminatum*, and *F. clamydosporum* species with frequencies 41.6, 31.3, 16.6, 6.6, and 3.3%, respectively, were isolated from the bean fields of Selseleh County, Lorestan province. The isolates were morphologically identified using Leslie and Summerell's identification key. In this study, 13 isolates of three *Fusarium* species, including *F. oxysporum*, *F. solani*, and *F. equiseti* were used to identify more precise with both morphological and molecular methods and also investigate their phylogenetic relationship with each other and with the reference species registered in the gene bank and ISTH. *TEF-1α* gene region was amplified using PCR, and the amplified fragments were sequenced. Nucleotide sequences were entered in *Fusarium* ID database for molecular identification of isolates based on the *TEF-1α* gene, and a phylogenetic tree was drawn. *F. solani* and *F. oxysporum* species were reported to be the most abundant and harmful *Fusarium* species causing bean root rot, which can be considered a limiting factor in bean cultivation in Selseleh County. Furthermore, the other species, which have low frequency and poor distribution, did not have a significant role in bean root rot in these fields.

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Introduction

Legumes are one of the essential and primary protein sources in the diet of humans and livestock worldwide. Bean (*Phaseolus vulgaris* L.) belongs to the family of *legumes* (*Fabaceae*). According to the report of the World Food and Agriculture Organization (FAO), the four countries of Myanmar, India, Brazil, and the United States of America are ranked first to fourth in production in 2020 (Canton, 2021). According to the Statistics and Information Technology Office of the Ministry of Agricultural Jahad report in the agricultural year of 1397-1398, legumes after cereals are the most important crops in Iran, with 7.1% of the total cultivated area of crops. Beans with a cultivated area of 106 thousand hectares account for 40% of the total production of legumes. Fars province has the first place in the area under cultivation and production of beans, followed by Lorestan, Zanjan, and Markazi provinces (Ahmadi *et al.*, 2020).

Among the factors that affect the performance of beans are plant pathogens, which cause different degrees of damage to this product (Burke, 1965). Among the diseases, fungal diseases are significant in this plant. Soil-borne fungi are one

of the most critical and dangerous factors. *Rhizoctonia solani* J.G. Kühni, *Pythium ultimum* Trow, *Macrophomina phaseolina* (Tassi) Goid, *Fusarium oxysporum* Schltdl., and *F. solani* (Mart.) Sacc. are the most critical soil-borne pathogenic fungi of beans. Other *Fusarium* species are *F. acuminatum* Ellis & Everh. *F. anthophilum* (A. Braun) Wollenw., *F. avenaceum* (Fr.) Sacc., *F. culmorum* (Wm.G. Sm.) Sacc. , *F. equiseti* (Corda) Sacc., *F. proliferatum* (Corda) Sacc., *F. redolens* Wollenw. and *F. crookwellense* L.W. Burgess, and P.E. Nelson & Toussoun reported pathogenic fungi causing bean root rot (Montiel-González *et al*., 2005). *Fusarium* genus is one of the world's most destructive toxin-producing plant pathogenic groups. These pathogens threaten food security through toxins. Historically, *Fusarium* classification has focused on the asexual (anamorph) as the sexual (telomorph) form is largely unknown for most species. Morphological characteristics of fungi are identified based on size, shape, and the presence or absence of asexual reproductive structures in different cultural environments (Leslie and Summerell, 2008). In the last two decades, phylogenetic species identification has identified about 400 phylogenetic species in *Fusarium*. Different descriptive guidelines (morphological and DNA-based) and databases, including FUSARIOID-ID, act as fast and accurate tools for identifying *Fusarium* species (Crous *et al*., 2021). The first step in controlling diseases caused by *Fusarium* is to accurately identify the species of this pathogenic fungus in bean fields.

Molecular methods are one of the most accurate tools for distinguishing between species and identifying new species collected from contaminated samples. Molecular methods have also been used to differentiate between closely related species with little or no morphological differences (Wulff *et al*., 2010) and to distinguish isolates (or even specific isolates) within a species (Chandra *et al*., 2011). *TEF-1α* gene encoding translation elongation factor 1α (*TEF-1α*) is used in sequence analysis for classification and phylogenetic studies (Geiser *et al*., 2004). The study of pathogen population identification is the basis of cultivar production and improvement programs to achieve resistant cultivars as the healthiest, most economical, and most effective methods of plant protection in the integrated management of pathogens (Silva *et al*., 2013; Hasanvand *et al.*, 2014). This study was conducted to isolate and identify *Fusarium* isolates in bean fields of Selseleh County based on molecular and morphological characteristics.

Material and Methods

Collection of *Fusarium* species

In the spring and summer of 2022, plants were sampled with signs of yellowness, stunted growth, leaf fall, and root and crown rot of bean fields in different areas of Selseleh County in Lorestan province. The samples were transported to the laboratory in clean plastic bags. The complete details of the sample, collection location, and date were recorded during sampling. In the laboratory, the root and crown of the samples were carefully washed with normal water, and then, from the border between the healthy tissue and the discolored parts of the root and crown pieces of 5-10 mm were cut and soaked in bleach liquid for 1-3 minutes. Commercial Vitex one percent (containing half percent of sodium hypochlorite active ingredient) was used to disinfect. The pieces were rinsed twice with sterile distilled water for one to three minutes and dried on sterile filter paper. In order to isolate the disease agent from the root and crown, the samples were cultured in potato-dextrose-agar culture medium and Nash and Snyder culture medium (Nash and Snyder) containing antibiotic amoxicillin and were kept in an incubator for seven days (Nash and Snyder, 1962). After the appearance of the fungal colony, the isolates were immediately transferred to the SNA (Spezieller Nährstoffarmer Agar) culture medium and gradually purified, using the single spore or thread tip method on two percent water agar (WA) medium (Leslie and Summerell, 2008).

One of the valid keys was used to identify *Fusarium* species, considering the most important morphological characteristics, including the type of phialid, the presence or absence of chlamydospores and microconidia, shape and size of macroconidia, and colony color (Booth, 1971; Leslie and Summerell, 2008; Aoki, 2012; Crous *et al*., 2021).

Molecular and phylogenetic analysis

DNA extraction was done using the CTAB method (Zhang *et al*., 2010). After DNA extraction, the PCR test was performed with a pair of primers related to the *TEF* gene and using the PCR Master Mix kit. Standard polymerase chain reaction was performed to amplify the *TEF-1α* gene region utilizing a pair of relevant primers including *ef*1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') as forward primer and *ef*2 (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3') was used as reverse primer for sequencing and phylogenetic analysis (Geiser *et al*., 2004). These primers amplify the 700 bp region of *TEF-1α* in *Fusarium*. The quantity and concentration of the polymerase chain reaction were prepared in the final volume of 25 µl, and after placing 0.2 ml microtubes in the ThermoCycler (BIORAD® T100TM, Germany), the thermal program was used for DNA replication in one cycle and primary denaturation for five minutes at 94 °C and then 35 cycles including denaturation at 94°C for 60 seconds, annealing of primers at 55 °C for 50 seconds and extension at 72 °C for three minutes and the final extension (one cycle) was at 72 °C for 10 minutes.

The resulting sequences from two forward and reverse sequences were edited using BioEdit software (BioEdit, 7.1). The obtained sequence data were deposited in the NCBI data set (GenBank, NCBI, USA; ([Online] http://www.ncbi.nlm.nih.gov/). The BLAST search using the edited sequences *TEF-1α* in the Gene Bank data set at NCBI database was performed to examine the highest similarity to aid in diagnosing and confirming *Fusarium* species.

In order to find the position and phylogenetic relationships of the identified species with each other and with other gene bank isolates, the phylogenetic tree of the isolates was drawn based on the latest changes in the genus *Fusarium* (Geiser *et al.,* 2021) using MEGA 7.0 software. For this purpose, first, the sequences of representative isolates (Table 1) and gene bank isolates (Table 2) were aligned with the Clustal W tool. The phylogenetic tree of *TEF-1α* sequences was drawn using the Maximum Likelihood algorithm and bootstrap 1000 repetitions. *Alternaria solani* (Ellis & G. Martin) and L.R. Jones & Grout was used as an outgroup to root the tree.

Table 1. The isolates sequenced in this study and their accession numbers obtained from the gene bank.

Isolate Names	Species	GenBank accession numbers of *TEF*-1α
IR:Be:Se1	*F. falciforme*	OQ117026
IR:Be:Se3	*F. solani*	OQ349681
IR:Be:Se4	*F. oxysporum*	OQ349682
IR:Be:Se5	*F. equiseti*	OQ366320
IR:Be:Se6	*F. oxysporum*	OQ366321
IR:Be:Se7	*F. oxysporum*	OQ372347
IR:Be:Se9	*F. solani*	OQ372348
IR:Be:Se11	*F. solani*	OQ385073
IR:Be:Se14	*F. falciforme*	OQ385074
IR:Be:Se15	*F. oxysporum*	OQ390198
IR:Be:Se17	*F. falciforme*	OQ390199
IR:Be:Se18	*F. oxysporum*	OQ390870
IR:Be:Se20	*F. equiseti*	OQ200421

Table 2. *Fusarium* species and their gene bank accession numbers related to other research used in this study.

Fungus	Accession number	Host	Strain	Country
Fusarium falciforme	MK752502.1	*Solanum tuberosum*	11A	Algeria
Fusarium solani	KF624788.1	Bean	B12	USA
Fusarium solani	MT371389.1	Bean	SMFS8	China
Fusarium solani	MT305241.1	*Asparagus root*	GR_FS243	Spain
Fusarium falciforme	ON375421.1	Bean	YC7-521	China
Fusarium solani	MK560290.1	Soybean	A9s6	China
Fusarium solani	MG973094.1	Tomato root	voucher WY12	Algeria
Fusarium oxysporum	MF327628.1	Tobacco	voucher 210	Argentina
Fusarium oxysporum f. sp. *cepae*	KP964904.1	Cepae	L9_1	UK
Fusarium nirenbergiae	MZ921870.1	Soil	JW 288013	Netherlands
Fusarium oxysporum f. sp. *cumini*	LT841203.1	Cumini	F11	Netherlands
Fusarium oxysporum f. sp. *lactucae*	MK801787.1	Lettuce	3_19	Italy
Fusarium oxysporum	KX822794.1	*Limonium sinuatum*	STAT9	U.K.
Fusarium oxysporum f. sp. *pisi*	KF913725.1	*Pisum sativum* L.	CBS 127.73	Spain
Fusarium oxysporum f. sp. *asparagi*	MT305190.1	*Asparagus root*	GR_FOA128	Spain
Fusarium oxysporum f. sp. *tulipae*	EU313536.1	*Tulipa* sp.	FoGa2	USA
Fusarium oxysporum f. sp. *vasinfectum*	EU246572.1	Cotton	IMI-325576	Australia
Fusarium solani	KY486685.1	Strawberry	Fs-148P	Spain
Fusarium solani	KX523136.1	*Casuarina sp.* L.	PVA-66	Mexico
Fusarium solani	MW366814.1	*Chickpea*	FsoCh8	USA
Fusarium solani	MN329690.1	*Citrus* spp.	IIIOF2	Brazil
Fusarium solani	ON366447.1	Tobacco	MC-4	China
Fusarium solani	MT371389.1	*Salvia miltiorrhiza*	SMFS8	China
Fusarium f. sp. *oxysporum*	MK675301.1	*Macrochloa tenacissima*	TN196	Tunisia
Fusarium equiseti	MT305067.1	*Asparagus* root	GR_FE153	Spain
Fusarium equiseti	MT152328.1	*Avena* sp.	A24	Iran
Fusarium clavum	MG826890.1	Wheat	wxwh40	USA
Fusarium clavum	MT104577.1	*Hordeum murinum*	H4R3	Iran
Fusarium equiseti	KR108318.1	*Lens culinaris*	FWJ47	Pakistan

Results

Identification of *Fusarium* species

A number of 95 samples of bean plant roots suspected of contamination and with symptoms of yellowness, wilting, and root rot and necrosis were examined from different parts of bean fields in Selseleh County, and 60 isolates of *Fusarium* spp. were isolated and identified based on morphological characteristics, from which 13 suspected isolates were used for more accurate identification by both morphological and molecular methods.

Fusarium oxysporum Schltdl.

The fungal colony grew rapidly on PDA medium at 25°C and reached 8.0± 1.0 cm in diameter after seven days. The myceliums were white to purple-white cotton, and the bottom surface of the colony was white to purple. Microconidia were often single-celled, ovoid, oval, or ovate, measuring 4.4-11.9× 2.1- 4.8 micrometers in false heads. One of the characteristics of this fungus was the production of many false heads on short monophialides. Macroconidia were mostly four-celled and were produced on pale orange cushions with dimensions of 21.2-44.2 × 3.4-6.11 micrometers. The terminal cell of the macroconidium was slightly curved and hook-shaped, and the basal cell was often foot-shaped. Chlamydospores were formed in abundance and with a diameter of 5.4-15.2 µm singly or in pairs after seven to 14 days in the culture medium (Fig. 1)

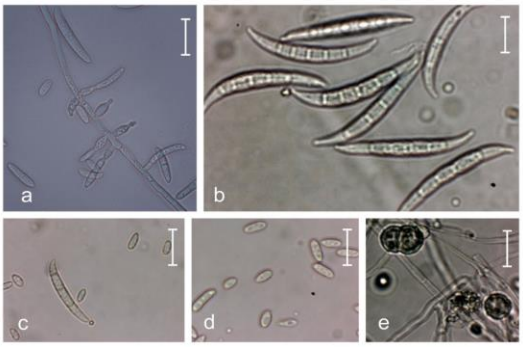

Fig. 1. Morphological characteristics of *Fusarium oxysporum*: a) monophialid, b) macroconidium, c) macro and microconidium, d) microconidium, e) chlamydospor (scale= 10µm).

***Fusarium solani* (Mart.) Sacc.**

The fungal colony grew rapidly on PDA medium at 25°C and reached a diameter of 6.5± 0.5 cm after seven days. Myceliums were sparsely grown and flat, often pale grayish-cream or white. Microconidia were abundantly formed on long phialides and in false heads. They are usually one-celled with dimensions of 4.18-1.6 × 1.5- 5.8 micrometers or two-celled with dimensions of 10.1- 28.14 × 2.6- 6.8 micrometers, ovoid, oval, or small. Macroconidia were produced with four or five cells, measuring 17.2- 60.78× 4.4-7.2 micrometers on white to cream-colored cushions. Chlamydospores were often seen singly or in pairs, in abundance, and with a diameter of 5.2-13.6 µm (Fig. 2).

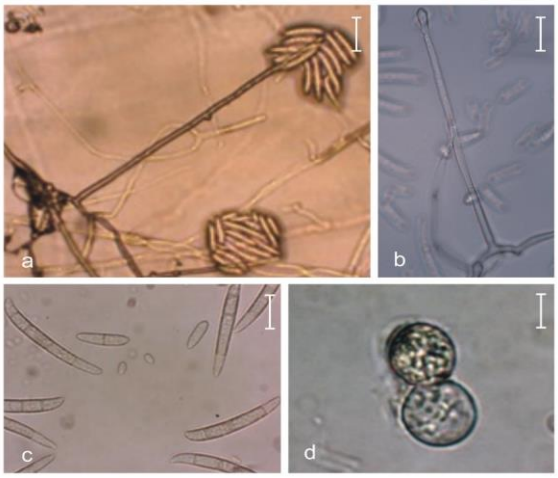

Fig. 2. Morphological characteristics of *Fusarium solani*: a) monophialid and false head, b) monophialid, c) macro and microconidium, d) chlamydospore (scale= 10µm).

***Fusarium equiseti* (Corda) Sacc.**

Fungal spores grew rapidly on PDA culture medium at 25°C and reached a diameter of 8.5± 0.5 cm after seven days. The myceliums are cottony and white in color, which turned into a dull brown color after some time. The isolates did not produce microconidia. Macroconidia are thick and have a lot of dorsal curvature. Macroconidia have a distinct foot-shaped basal cell and a narrow terminal cell that may be filamentous or whip-like. These conidia have four or five cells, measuring 31.4-51.9 × 4.3-5.6 micrometers, and were produced on orange cushions. Chlamydospores were formed abundantly and in clusters or chains (Fig. 3).

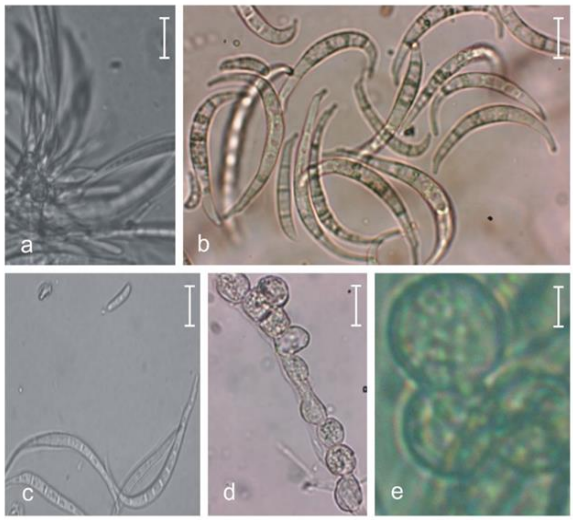

Fig. 3. Morphological characteristics of *Fusarium equiseti*: a) monophialides, b) macroconidium c) macro and microconidium, d and e) chlamydospores (scale= 10µm).

***Fusarium acuminatum* Ellis & Everh**

The fungal colony grew relatively slowly on the PDA culture medium at 25 °C and reached a diameter of 6.0± 0.5 cm after seven days. The myceliums were cottony and grayish-white in color. The isolates did not produce microconidium and chlamydospores. Five or six-celled macroconidia with moderate curvature and dimensions of 31.6- 48.4 × 3.3 × 5.4 micrometers were produced on orange cushions. Also, macroconidia have a thick cell wall and foot-shaped basal cells distinct from the cell, although not as much as *F. equiseti* species (Fig. 4).

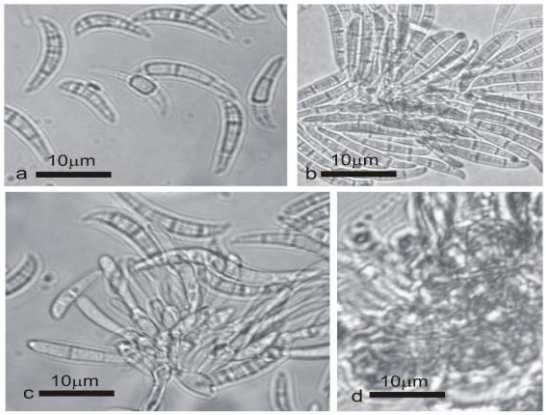

Fig. 4. Morphological characteristics of *Fusarium acuminatum*: a,b) macroconidium; c) monophialides; d) chlamydospores (scale= 10µm).

***Fusarium chladosporum* Wollenweber & Reinking**

Fungal colonies on the PDA culture medium were mostly pale grayish-white. Microconidia were often comma-shaped on both single and polyphialides. Macroconidia have a thick wall and moderate curvature; the upper wall is curved, the lower wall is almost straight, and usually five or six cells are produced on orange cushions. Chlamydospores were often formed in chains or clusters, abundantly and rapidly. Production of chlamydospores by old cultures caused mycelium to have a pale brown color (Fig. 5).

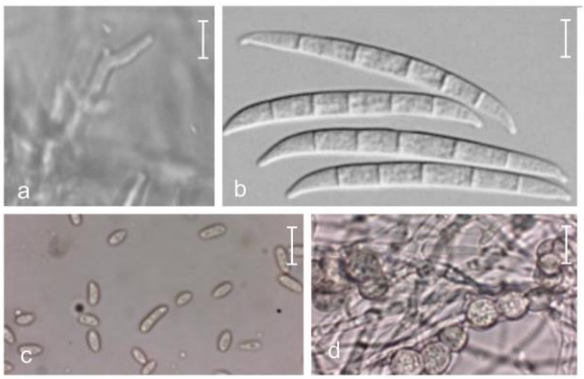

Fig. 5. Morphological characteristics of *Fusarium chladosporum*. a,b) morphology of macroconidium; c) microconidium; d) chlamydospore (scale= 10µm).

Molecular identification and phylogenetic analysis of *Fusarium* species

In the PCR test, the primer pair ef1/ef2 successfully amplified a 700 bp fragment containing the *TEF-1α* gene region in 13 selected isolates of *Fusarium* spp. (Fig. 6). The isolates in this study were placed in three main groups in the phylogenetic tree. The first group includes all isolates of *F. oxysporum* and also the isolates Se18, Se15, Se7, Se6, and Se4. Using the BLAST tool in the NCBI database showed this group has the most similarity with isolate MZ921870 (*F. nirenbergiae*), and they were placed in the same branch. The second group includes all isolates of *F. equiseti*, including the isolates Se5 and Se20. This group was the most similar to isolates MG826890.1 and MT104577 (*F. clavum*) and were placed in the same branch. The third group includes all isolates of *F. solani* and *F. falciforme*, including the isolates Se3, Se11, and Se9 (*F. solani*) and Se1, Se14, and Se17 (*F. falciforme*) were placed in one branch (Fig. 7). *Alternaria solani* species was considered as outgroup to root the tree.

Discussion

Fusarium species are important fungi that cause bean root rot (Montiel-González *et al*., 2005). In this study, 60 isolates of the genus *Fusarium*, including *F. solani*, *F. oxysporum*, *F. equiseti*, *F. acuminatum*, and *F. clamydosporum* species were isolated and identified with frequencies of 41.6, 31.3, 16.6, 6.6, and 3.3 percent, respectively, in the bean fields in Selseleh County. Thirteen suspected isolates, including *F. oxysporum*, *F. solani*, and *F. equiseti*, were selected and investigated for more accurate identification by both morphological and molecular methods. This disease causes significant damage to farmers every year in Iran. The damage to the crop is estimated to be up to 85 percent in infected areas (Ahari *et al*., 2009).

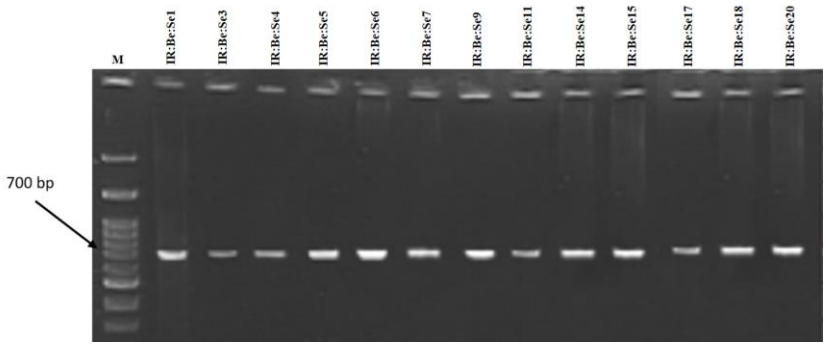

Fig. 6. Amplified fragments of *TEF-1α* gene region in the 1% agarose gel electrophoresis: M= 100bp marker, IR:Be: Se1- IR:Be: Se20= indicate isolate names.

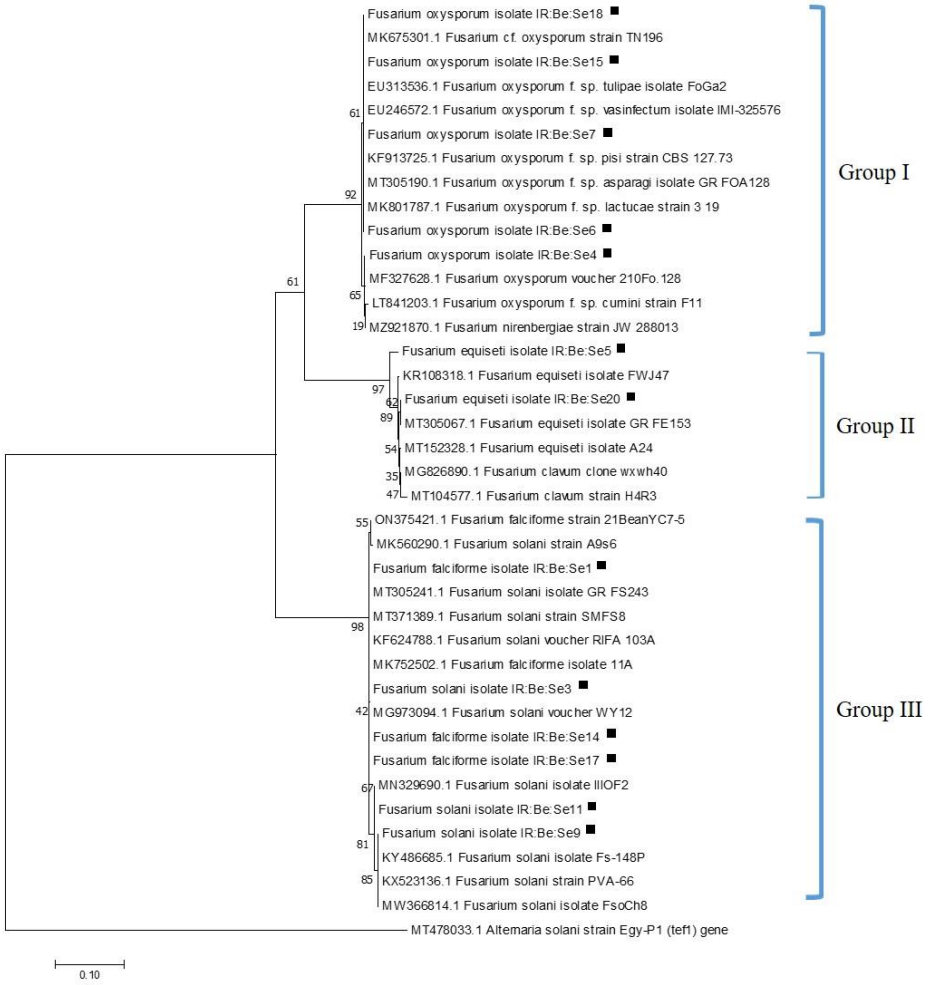

Fig. 7. The phylogentic tree of the isolates *Fusarium* spp. based on the *TEF-1α* gene region sequence drawn by MEGA 7.0 with the maximum likelihood method and bootstrap values of 1000 replicates at the branch point. Selected isolates in this research are marked with black squares.

This fungus has been introduced as one of the most important factors causing damage to bean fields with the highest abundance in Zanjan, Qazvin, East Azerbaijan, and Lorestan provinces in the past (Naseri, 2008; Saremi *et al*., 2011; Dehghani *et al*., 2019). Dehghani *et al.* (2019) isolated and reported the species *F. solani* and *F. oxysporum* from Lorestan province with an abundance of 33.42 and 26.55, respectively. In studies done by Saremi *et al.* (2011) and Dehghani *et al.* (2019) in Lorestan, Qazvin, East Azerbaijan, and Zanjan provinces *F. oxysporum* was after *F. solani* in terms of abundance (Saremi *et al*., 2011; Dehghani *et al*., 2019). In the classification of Crous *et al.* (2021), the species complex *F. solani* was merged into the species of the genus *Neocosmospora*, and all the species identified by Aoki were merged into the species *Neocosmospora phaseoli* (Crous *et al*., 2021). *Neocosmospora*, as a group of soil-borne fungi, are often associated with roots and cause root rot and vascular wilt diseases. In addition to dissimilar sexual characteristics, such as purple or black perithecium and asexual form, this group has been merged with other *Fusarium* species. Macroconidia usually have a thick wall, smooth or round terminal cells, and typically have foot-shaped basal cells. Microconidia are produced on very long and narrow phialides. This merging of traits in the genus *Neocosmospora* may occur due to the horizontal transfer of genes that increase the ability of a pathogen to adapt to host defense mechanisms and allow organisms to acquire ecologically

beneficial genes (Crous *et al*., 2021). There are several reasons for the diversity of *Fusarium* isolates. In *Fusariums*, mutations, sexual reproduction, and heterokaryon formation through vegetative adaptation and the prosexual cycle are the most important factors of genetic structure changes (Summerell *et al*., 2001). Although the sexual stage of *Fusarium* fungus from beans has not been reported in Iran, seven biological groups (I-VII) or compatible mating types have been identified in terms of sexual reproduction in *F. solani* (Matuo and Snyder, 1973). Species of the genus *Fusarium* have changed many of their biological characteristics under constant evolutionary pressure to adapt better and occupy different ecological systems (Desjardins *et al*., 1993). *F. equiseti* and *F. acuminatum* species were isolated and identified with low frequency from the fields. Safarloo and Hemmati (Safarloo and Hemmati, 2014) isolated *F. acuminatum* with an abundance of 8 percent in Zanjan province. Dehghani *et al.* (2019) identified this fungus as one of the species with low abundance in Lorestan province. This fungus is probably of little importance in bean fields in Iran. There are several reports of *F. equiseti* in *legumes* (Rahkhodaei *et al*., 2021; Safarloo and Hemmati, 2014). This fungus has been reported as the cause of bean root rot (Asan, 2011). The low abundance and poor distribution of *F. equiseti* in bean fields indicate that their role in bean root rot is not very important (Montiel-González *et al*., 2005). Mainly, *F. equiseti* is considered a saprophyte (Leslie and Summerell, 2008). The first step to control the disease is to identify the pathogen. Identification of *Fusarium* species is necessary to determine the cause of root diseases in legumes and manage the disease suppression (Zhou *et al*., 2021). Since ITS region sequences cannot distinguish *Fusarium* species exactly, based on the characteristics of the *TEF-1α* gene region, it is preferred to use the *TEF-1α* gene region to identify *Fusarium* species, which can provide useful and more accurate information in distinguishing *Fusarium* species. In addition to ITS and *TEF-1α* gene regions, other genes such as rpb2, act1, and tub2 are also used in phylogenetic studies of *Fusarium* species (Geiser *et al*., 2021).

Conclusion

The traditional harvesting of beans and placing plant seeds and organs on the surface of the contaminated soil for several days and then collecting them are some of the most important ways of transferring pollution between farms. Cultivation of potatoes, legumes, and various types of plants in different years in a contaminated farm and the transmission of contaminated seeds of legumes such as chickpeas, mung beans, and soybeans increase the possibility of the role of gene flow between the isolates in the fields. According to the results of this research in Selseleh County, *F. solani* and *F. oxysporum* species are the most abundant and harmful *Fusarium* species that cause bean root rot, which can be considered as a limiting factor in bean cultivation, and to reduce their damage, integrated management and the use of resistant cultivars should be considered. Therefore, future studies with more isolates and genes and the use of molecular markers will not only determine the genetic similarities of the isolates but also make it possible to distinguish new species. On the other hand, the study of population genetics of *Fusarium* isolates leads to a better view of control methods, especially the use of cultivars.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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