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ORIGINAL ARTICLE

Morphological, molecular and pathogenic characterization of *Fusarium* spp. associated with chickpea wilt in western Iran

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Abstract

Fusarium wilt is one of the most severe diseases of chickpea in the major growing areas of chickpea production in western Iran. To identify *Fusarium* spp. associated with chickpea plants showing symptoms of yellowing and wilting, 58 chickpea fields were sampled and 106 *Fusarium* spp. isolates were obtained from six different regions of Kermanshah Province in western Iran during 2018 and 2019 crop seasons. Thirty-six isolates obtained from stem or lower stem tissues were selected for pathogenicity, morphological and molecular identification using polymease chain reaction species-specific primers. Eleven isolates of *Fusarium* spp. were selected for sequence analyzing the translation elongation factor 1-α (*EF-1*α), and *β-tubulin* gene regions. Phylogenetic analysis of concatenated DNA sequences of both gene regions of these isolates plus other taxa revealed that 11 *Fusarium* spp. isolates were clustered into five distinct groups. Based on the results of morphological and molecular identification five *Fusarium* species were identified. Pathogenicity tests showed that *F. oxysporum* f. sp. *ciceris* and *F. redolens* isolates had the highest disease incidence on JG–62 and Bivenij cvs. and *F. hostae, F. equiseti* and *F. acuminatum* isolates had the lowest disease incidence. No sign of vascular discoloration was observed in longitudinal or transverse sections of chickpea plants affected by *F. redolens* isolates. Instead, brown to black necrosis was observed on the surface of tap-roots and crowns. No correlation was found between geographical distribution and pathogenicity of isolates. This is the first report of morphological, molecular and pathogenicity characteristics of *F. redolens* and *F. hostae* isolated from chickpea stems or lower stems in Iran.

Keywords: *Cicer arietinum*, chickpea wilt, *Fusarium redolens*, *Fusarium hostae*

Introduction

Chickpea (*Cicer arietinum* L.) is the third most important grain food legume in the world after dry bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.; Sharma and Muehlbauer 2007) and it is the most important pulse crop in Iran. Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend: Fr. f. sp. *ciceris* (Padwick) T. Matuo & K. Satôis is the most severe disease of chickpea after Ascochyta blight in the major growing areas of its production in western and northwesern Iran. The disease causes severe crop losses of chickpea yield of up to 100% under favorable conditions (Haware and Nene 1982). Although the crop losses due to this disease in Iran have not been estimated yet, the average yield loss is commonly about 10–12% (personal observations). The pathogen can survive as mycelium and chlamydospores in seed and soil and also on infected crop residues, roots and stem tissue buried in the soil for more than 6 years, even in the absence of the host (Jendoubi *et al.* 2017).

Two pathotypes have been distinguished within *F. oxysporum* f. sp. *ciceris* based on the distinct yellowing or wilting syndromes they cause in chickpeas (Trapero-Casas and Jiménez-Díaz 1985). The yellowing pathotype induces progressive foliar yellowing

with vascular discoloration, while the wilting pathotype induces severe and fast chlorosis, flaccidity and vascular discoloration. In addition to major symptom types, eight pathogenic races (races 0, 1A, 1B/C, 2, 3, 4, 5 and 6) can be identified in *F. oxysporum* f. sp. *ciceris* (Haware and Nene 1982; Jiménez-Gasco *et al.* 2001). Races 0 and 1B/C induce yellowing symptoms (yellowing pathotype), whereas the remaining races cause wilting (wilting pathotype) symptoms (Jiménez-Gasco *et al.* 2001, 2003).

A study conducted by Jiménez-Fernández *et al.* (2011) revealed that infection of chickpea by *F. redolens* induces a disease syndrome similar to that caused by the yellowing pathotype of *F. oxysporum* f. sp. *ciceris*. *Fusarium redolens* is not easily differentiated from *F. oxysporum* f. sp. *ciceris* using morphology-based diagnosis, and the two species cause similar symptoms on chickpea. Therefore, the use of molecular protocols should help to avoid misdiagnoses of Fusarium yellows in chickpea (Jendobi *et al.* 2017). The use of DNA-based methodologies has recently made it possible to clearly differentiate between these two species. It has also been revealed that *F. redolens* and *F. oxysporum* species complex lack a sister group relationship (Bogale *et al.* 2007). Bogale *et al.* (2007) developed a species-specific primer to identify *F. redolens* isolates. This *F. redolens*-specific amplification product differentiated *F. redolens* from other *Fusarium* spp. and allowed rapid and simple diagnoses of *F. redolens* isolates.

Fusarium wilt of chickpea has been reported in almost all chickpea growing areas of the world (Sharma and Muehlbauer 2007). In Iran, Manuchehri and Mesri (1966) first reported the occurrence of the disease and introduced *F. lateritium* f. sp. *ciceris* as its causal agent. After that, several reports of disease occurrence and pathogenic and genetic variability of the pathogen were published (Afshari Azad 1998; Younesi 2004; Mohammadi and Banihashemi 2005; Zokaee *et al.* 2012; Nourollahi *et al.* 2017; Chehri 2018; Younesi *et al.* 2019). Initial reports were mainly based on morphological characteristics. Except for the first report, in other reports, *F. oxysporum* f. sp. *ciceris* has been considered as the primary pathogen of chickpea Fusarium wilt. Recently, Saeedi and Jamali (2021) reported that the frequency of *F. redolens* in uncultivated soils was more than other species. In addition, all isolates obtained from the roots of chickpea plants with black root rot symptoms were identified as *F. redolens* based on morphological and molecular characteristics. They concluded that *F. redolens* also can be the causal agent of chickpea black root rot in other parts of Iran (Saeedi and Jamali 2021). Pathogenic diversity in *F. redolens* resembles that in *F. oxysporum* and isolates of *F. redolens* have been associated with a wide range of diseases, including wilts, seedling damping-off, and cortical rot (Baayen *et al.* 2011). So, the association of *F. redolens* with symptoms of yellowing and wilting disease of chickpea is little understood.

The first aim of this study was to identify *Fusarium*-spp. isolated from stems and lower stems of chickpea plants showing symptoms of yellowing and wilting and their pathogenicity on chickpea cultivars. In addition, some attempts were made to study the association of *F. redolens* and *F. hostae* with symptoms of yellowing and wilting of chickpea plants. The results of this study can be useful in decision-making of management strategies for chickpea Fusarium wilt and provides some new information about the *Fusarium* species associated with chickpea wilt in Iran.

Materials and Methods

Sampling

Major chickpea cultivation areas of Kermanshah province in western Iran were visited in five different geographic directions and chickpea fields were sampled at 5 km intervals. Sampling was done by cutting a segment 4–6 cm long from the upper part of the main chickpea stem with yellowing and wilting symptoms, with or without browning of xylem tissues. The samples were kept in polyetilen bags and were transferred to the laboratory. Each 4–6 cm piece was cut into 0.5–1.0 cm pieces which were surface-disinfested with sodium hypochlorite (NaOCl 0.5%) for 2 min, washed with sterile distilled water twice, and dried on sterile filter paper. Then, root pieces were placed on a *Fusarium* selective and non-selective media and kept at 25 ± 1°C and 12-h photoperiod of fluorescent and Near-UV at 2000 Lux for sufficient growth over 5 days. Single spore isolates were stored in slant tubes containing potato dextrose agar (PDA) (Difco Laboratories, Detroit) for short-term storage at 4°C in a refrigerator. For long term maintenance, 2 ml of conidial suspension (1×10^6 spores · ml^{-1}) of each isolate were mixed in 10 g of sterilized soil : sand (1 : 1 v/v) mixture and kept at 4°C in a refrigerator. For reusing, a small amount of contaminated soil was placed on the PDA and was used after appropriate growth.

Morphological identification of *Fusarium* spp.

Morphological identification of *Fusarium* spp. was done based on cultural, macroscopic and microscopic characters according to Nelson *et al.* (1983) and Leslie and Summerell (2006). Macroscopic characteristics included colony appearance, size, color, growth rate and other characteristics. Microscopic characters, including shape, color and size of microconidia, macroconidia and chlamydospores, were examined under a calibrated compound microscope (Olympus, Model:

BH-2, Japan). For the assessment of growth rate, 5 mm discs were cut from actively growing edges of each colony and placed on PDA plates to be incubated at 25 ± 1°C with a 12/12 h photoperiod. The colony diameter of each isolate was measured at 24 h intervals up to 10 days. Four replicates were maintained for each isolate/medium. The mean of 50 observations for each character was recorded and the maximum, minimum and average sizes were calculated.

Molecular identification

DNA extraction

Fusarium isolates were grown on sterile cellophane film on PDA for 7 days at 25 ± 1°C. About 100–200 mg of mycelia were scraped off using a sterile scalpel and after drying by liquid nitrogen, were broken using a mortar and pestle. Crashed mycelia were harvested and fungal DNA was extracted by using a commercially available DNA extraction kit (Genomic DNA isolation kit IV; DENA Zist Asia, Iran) based on the manufacturer's protocol. DNA yield was measured by the absorption rates of samples in 260 and 280 nm with a spectrophotometer. The quality of extracted DNA was evaluated using 2% agarose gel electrophoresis. Agarose gel was electrophoresed at a constant voltage of 104 mA for 25 min. It was then viewed and photographed under UV light in a Gel Doc transilluminator (Advanced Biotech Company, Kiagen, Iran).

Characterization of *Fusarium* spp.

PCR-specific species primers FoF1/FoR1, Foc012f/Foc012r and Redolens-F/Redolens-R developed by Mishra *et al.* (2003), Jiménez-Gasco and Jiménez-Díaz (2003) and Bogale *et al.* (2007), respectively, were used to confirm identification of *F. oxysporum*, *F. oxysporum* f. sp. *ciceris* and *F. redolens* isolates based on morphological characteristics.

Partial sequences of the translation elongation factor 1-α (*EF-1*α), and *β-tubulin* gene regions of 11 selected isolates were amplified by using primer pairs of EF1/EF2 (O'Donnell *et al.* 1998) and T1/T22 (O'Donnell and Cigelnik 1997) and a PCR thermocycler machine (PeQSTAR 96X Universal Gradient). PCR conditions for EF1/EF2 were provided as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 55°C for 50 s, 72°C for 3 min and a final extension of 72°C for 10 min (Wang and Zheng 2012). PCR conditions for *β-tubulin* gene with T1/T22 primers were: initial denaturation at 94°C for 5 min, followed by 35–40 cycles of 35 s at 94°C, 55 s at 52°C, and 2 min at 72°C and a final extension of 72°C for 10 min (O'Donnell and Cigelnik 1997).

All primers were purchased from Kaigen company, Iran. The resulting products were sequenced using an automated sequencer. Sequence editing and alignment were done using Bioedit software. The sequences were sent to the GenBank and accession numbers were obtained. *Fusarium* species were identified by searching in BLAST on the NCBI and FUSARIUM-ID sites and comparing with valid sequences. The right outgroup was chosen from the BLAST search. A phylogenetic tree was created by concatenated DNA sequences of *EF-1*α and *β-tubulin* gene regions and the maximum parsimony method with 1000 bootstrap using MEGA X software.

Pathogenicity tests

To examine the pathogenicity of *Fusarium* isolates, chickpea seeds of two highly susceptible chickpea cvs. JG-62 and Bivenij were disinfested with 0.25% sodium hypochlorite (NaOCl 0.25%) for 5 min. Then they were sown in polyethylene bags containing sterilized fine riverbed sand at 121°C for 2 consecutive days. Each *Fusarium* spp. *inocula* was propagated on autoclaved cornmeal sand (CMS) mixture (95 : 5 w/w) for 2 weeks at 25 ± 1°C with a 12/12 h photoperiod according to Nene and Haware (1980). One hundred grams of each inoculated CMS was mixed with 2 kg sterilized potting soil mixture (sand : soil 1 : 1) in 15 cm pots (Nene and Haware 1980). Five 7-day-old seedlings were removed from the sterilized fine riverbed sand and sown in pots with infested soil. The pots were watered as needed and fed nutrient solution at 1-week intervals. The pots were kept under greenhouse conditions at 22–28°C, 60–90% relative humidity and a 14-h photoperiod of fluorescent light.

The percentage of seedling germination was scored at 15 days after sowing of chickpea cultivars. The disease severity was recorded based on the percentage of foliage with yellowing and necrosis symptoms for each plant at weekly intervals for more than 60 days after planting using a 0 to 4 rating scale proposed by Navas-Cortes *et al.* (2000). The scale was as follows: 0 – 0%, 1 – 1 to 33%, 2 – 34 to 66%, 3 – 67 to 100% and 4 – death plant. The disease intensity index (Navas-Cortes *et al.* 2000) was calculated based on the following formula:

$$DII = \sum \frac{Si \times Ni}{Nt \times 4} \times 100,$$

where: *DII* – the disease intensity index, *Si* – wilt severity, *Ni* – the number of plants, *Nt* – the total number of plants investigated. Eventually the seedlings came out of the soil. Excess soil was removed by rinsing. Plants were examined for symptoms on their roots and the extent of discoloration of the vascular tissues was evaluated based on 0–4 scales provided by Trapero-Casas and Jiménez-Díaz (1985). Pathogeniciy experiments were set up in a completely randomized design. Data were analyzed using single factor analysis of variance

Table 1. Primers used for polymerase chain reaction (PCR) and sequencing of *Fusarium* spp. isolates

Primer	Sequence (5′ to 3′)	Reference
ef1	ATGGGTAAGGARGACAAGAC	O'Donnell *et al.* (1998)
ef2	GGARGTACCAGTSATCATGTT	
FoF1	ACATACCACTTGTTGCCTCG	Mishra *et al.* (2003)
FoR1	CGCCCATCAATCAATTTGAGGAACG	
Foc012f	GGCGTTTCGCAGCCTTACAATGAAG	Jiménez-Gasco and Jiménez-Díaz (2003)
Foc012r	GACTCCTTTTTCCCGAGGTAGGTCAGAT	
Redolens-F	ATC GAT TTTCCC TTC GAC TC	Bogale *et al.* (2007)
Redolens-R	CAATGATGATTGTGATGA GAC	
Tub2-T1	AACATGCGTGAGATTGTAAGT	O'Donnell and Cigelnik (1997)
Tub2-T22	TCTGGATGTTGTTGGGAATCC	

(ANOVA). Comparison of means was carried out using the Least Significant Difference (LSD) at the level of $p \leq 0.05$. Statistical analysis was performed with Statistical Analyzed System (SAS; version 9.1.3) software.

Results

A total of 58 chickpea fields were visited and 106 *Fusarium* spp. isolates were collected from six different regions of Kermanshah province in western Iran during 2018 and 2019 crop seasons. Thirty-six isolates obtained from either stem or lower stem tissues were selected for pathogenicity, as well as morphological and molecular identification. Sampling areas included Kermanshah (16 fields), Islamabad (14 fields), Ravansar (8 fields), Sahneh (6 fields), Kangavar (6 fields) and Harsin (8 fields).

Morphological identification

Thirty-six *Fusarium* spp. isolates belonged to five different species, based on morphological characters, according to Nelson *et al.* (1983) and Leslie and Summerell (2006). *Fusarium oxysporum* with 19 isolates (52.78%) and *F. redolens* with 12 isolates (33.33%) were the most abundant species isolated. *Fusarium equiseti*, *F. acuminatum* and *F. hostae,* with two isolates (5.56%), two isolates (5.56%) and one isolate (2.78%) frequencies, respectively, had the least abundance. *Fusarium oxysporum* isolates produced sparse to abundant white to pink white mycelia on PDA. These isolates produced pigmentation ranging from pale pink to cream in the agar at the bottom of the plate (Fig. 1). The growth rate of *F. oxysporum* isolates on PDA medium was 6.5–7.5 cm after 7 days at 25 ± 1°C. Macroconidia formed in pale orange spordochia after 7–10 days with straight to slightly curved thin walls, usually 3 septa and occasionally 4–5 septa with tapering and curved apical cell and foot shaped basal cell. Microconidia were produced abundantly in false heads on short monophialids on SNA, usually 0 – septate, and oval, elliptical, straight to kidney shaped. Chlamydospors formed abundantly, singly or in pairs, terminally or intercalary with smooth or rough walls (Table 2).

Fusarium redolens isolates showed abundant white aerial mycelia and pale brown pigmentation in agar at the bottom of the colony (Fig. 1). The growth rate of *F. redolens* isolates on PDA medium was the same as *F. oxysporum* isolates and varied from 6.5 to 7.5 cm after 7 days at 25 ± 1°C. Microconidia formed in false heads on short monophilalids on Synthetic Nutrient--poor Agar (SNA), oval to cylindrical, 0–1 septate and often pointed on one end. Macroconidia formed in small and cream to pale brown spordochia with 3–5 septa with hooked apical cell and foot shaped basal cell. Chlamydospors formed abundantly on Carnation Leaf Agar (CLA) after 30–40 days usually in short chains, singly or in pairs, terminaly or intercalary, and were spherical with rough walls (Table 2).

Fusarium hostae isolates exhibited limited aerial mycelia. The pigmentation of the agar at the bottom of the plate was brownish orange (Fig. 1). The growth rate of *F. hostae* isolates on PDA was 5.5–6.0 cm after 7 days at 25 ± 1°C. Macroconidia formed uniformally in orange sporodochia usually with 3 septa with curved or hooked apical cell and foot shaped basal cell. Microconidia were fusiform to falcate usually 0 – septate, but may be 1–2 septate. Sporulation in aerial mycelium on CLA formed from short, flask-shaped monophialides and occasional polyphialides (Table 2, Fig. 1). Chlamidospors formed abundantly on CLA, singly, in chains or in clumps (Leslie and Summerell 2006). *Fusarium hostae* can be distinguished from *F. redolens* based on the production of polyphialides, slower growth rates at 25°C and 30°C on PDA, a greater level of pathogenicity on hosta, and an analysis of DNA sequences (Geiser *et al.* 2001).

Fusarium equiseti isolates which exhibited abundant aerial mycelia were initially white and then

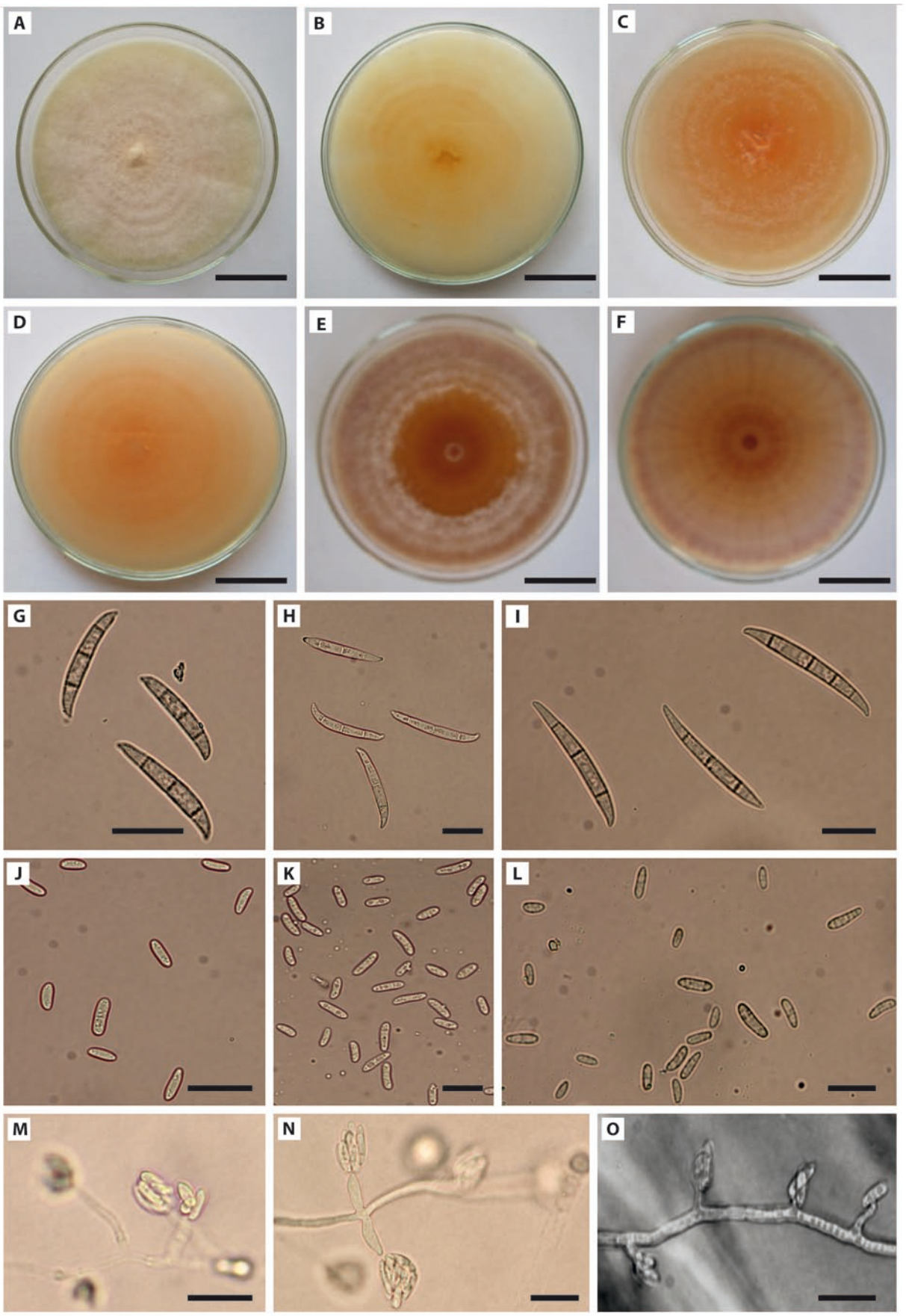

Fig. 1. Cultures and morphology of *Fusarium* spp.; (A–B) – colony appearance of *F. oxysporum* at 25°C, 7 days on PDA (A: front; B: bottom); (C–D) – colony appearance of *F. redolens* at 25°C, 7 days on PDA (C: front; D: bottom); (E–F) – colony appearance of *F. hostae* at 25°C, 7 days on PDA (E: front; F: bottom); (G–I) – macrocnidia: (G) – *F. oxysporum*; (H) – *F. redolens*; (I) – *F. hostae;* (J–L) – microconidia: (J) – *F. oxysporum*; (K) – *F. redolens*; (L) – *F. hostae*; (M–O) – short phialids false head: (M) – *F. redolens*; (N) – *F. hostae*; (O) – *F. oxysporum*; Bars A–F – 20 mm, G–O – 10 µm

Table 2. Morphological characters of *Fusarium* spp. isolates obtained from stem and lower stem parts of chickpea plants showing yellowing and wilting symptoms from major chickpea growing areas in western Iran

Fusarium species	Chlamido-spore diameter [µm]	Microconidia			Macroconidia				Sporo-dochia color
		size [µm]	shape	phialide	size [µm]	shape	apical	basal	
F. oxysporum	8.9–9.3	4.0–11.2 × × 2.1–3.2 (0 spt)	ellipsoid or reniform	short mono	25–52 × × 3.0–5.3 (3–5 spt)	straight to slightly curved	tapered or curved	foot shaped	pale orange
F. redolens	6.5–9.0	4.9–12.2 × × 2.6–5.2 (0–1 spt)	oval or cylindrical	short mono	25.5–44.1 × × 4.6–6.5 (3–5 spt)	robust	hooked	foot shaped	cream
F. hostae	6.2–9.1	4.9–13.0 × × 2.2–4.0	fusiform to flacate	mono and poly	33– 53.5 × × 4–5	falcate to fusiform	curved or hooked	foot shaped	purplish orange
F. equiseti	8.1–9.0	absent	absent	absent	32–65 × × 2.5–6 (3–7 spt)	slender and long	tapered and elongate	well developed foot shaped	orange
F. acuminatum	absent	absent	absent	absent	33–56 × × 3.1–5.2 (3–7 spt)	slender and falcate	tapered curved	well developed foot shaped	pale brown

spt = septate

turned brown. The pigmentation of the agar at the bottom of the colony was pale brown and occasionally dark brown spot pigments formed in the agar. The growth rate of *F. equiseti* isolates on PDA was 6.8 cm after 10 days at 25 ± 1°C. Macroconidia formed uniformally in orange sporodochia with usually 5–7 septa with whip-like apical cell and an elongated prominent foot shaped basal cell. Microconidia were absent and chlamidospors formed abundantly on CLA, singly, in chains or in clumps (Table 2).

Fusarium acuminatum isolates produced floccose aerial mycelia on PDA which were white to pale red in color. Pigmentation was deep red at the bottom of a colony. Macroconidia were 3–5 septa with relatively elongate apical cell and distinct foot-shaped basal cell. The growth rate on PDA was 7 cm after 10 days at 25 ± 1°C (Table 2).

Molecular identification

PCR-specific species primers FoF1/FoR1, Foc012f/Foc012r and Redolens-F/Redolens-R were used to confirm the detection of *F. oxysporum, F. oxysporum* f. sp. *ciceris* and *F. redolens* isolates which had been previously identified based on morphological characteristics. Based on estimates by agarose gel electrophoresis, specific primers of FoF1/FoR1, Foc012f/Foc012r and Redolens-F/Redolens-R produced products with about 1503, 340 and 386 bp for all *F. oxysporum*, *F. oxysporum* f. sp. *ciceris* and *F. redolens* isolates (Fig. 2). Other than the positive control isolate, no other *Fusarium* spp. provided the target band.

Eleven strains of *Fusarium* spp. were used to analyze the *EF-1*α and *β-tubulin* gene regions sequencing. The amplification of their regions with primer pairs of ef1/ef2 and T1/T22 produced as large as 700-bp and 1400-bp in electrophoresis agarose gel from those isolates (Fig. 2). By comparison of the sequences obtained from *EF-1*α and *β-tubulin* gene regions with the sequences gained from GenBank and FUSARIUM-ID, all *Fusarium* isolates were identified at the species level. Sequence analysis showed that the two isolates (IKFU7 and IKFU21) belonged to *F. oxysporum* f. sp. *ciceris* and six isolates (IKFU8, IKFU12, IKFU16, IKFU17, IKFU23 and IKFU26) were identified as *F. redolens*. Three isolates which belonged to *F. acuminatum*, *F. equiseti* and *F. hostae* each included a single isolate (Fig. 3). These results were in agreement with morphological and species specific-PCR identifications.

Phylogenetic relationships between the strains

The results of phylogenetic analysis from concatenated DNA sequences of *EF-1*α and *β-tubulin* gene regions showed that these 11 isolates of *Fusarium* spp. can be divided into five distinct groups (Fig. 3). In the first group, six isolates (IKFU8, IKFU12, IKFU16, IKFU26, IKFU23 and IKFU17) were identical to *F. redolens* FRLp. These isolates were isolated from the upper parts of chickpea stems, with yellowing and wilting symptoms and without discoloration of the vascular tissues. Isolate IKFU32 was closely related to strain *F. hostae* NRRL 9888 and classified into the second group. The third group including IKFU7 and IKFU21 isolates were related to *F. oxysporum* f. sp. *ciceris* NRRL 29888. Although phylogenetic analysis separated the isolates belonging to this group into two subgroups, it

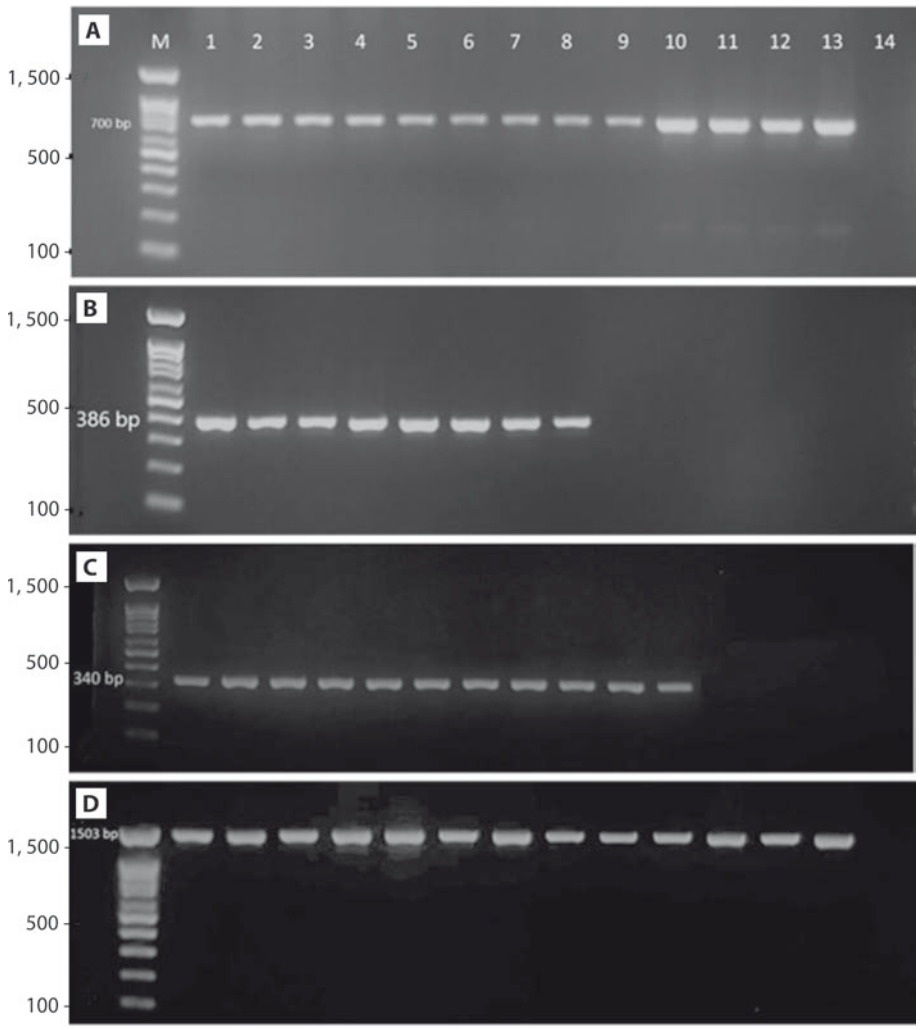

Fig. 2. Electrophoresis gel of DNA amplicons using: (A) – tef1-α region (700 bp), (B) – *Fusarium redolens* – specific primers (386 bp), (C) – *F. oxysporum* – specific primers (340 bp), (D) – *F. oxysporum* f. sp. *ciceris* – specific primers (1503 bp); M – 100 bp DNA Ladder (1,500 bp)

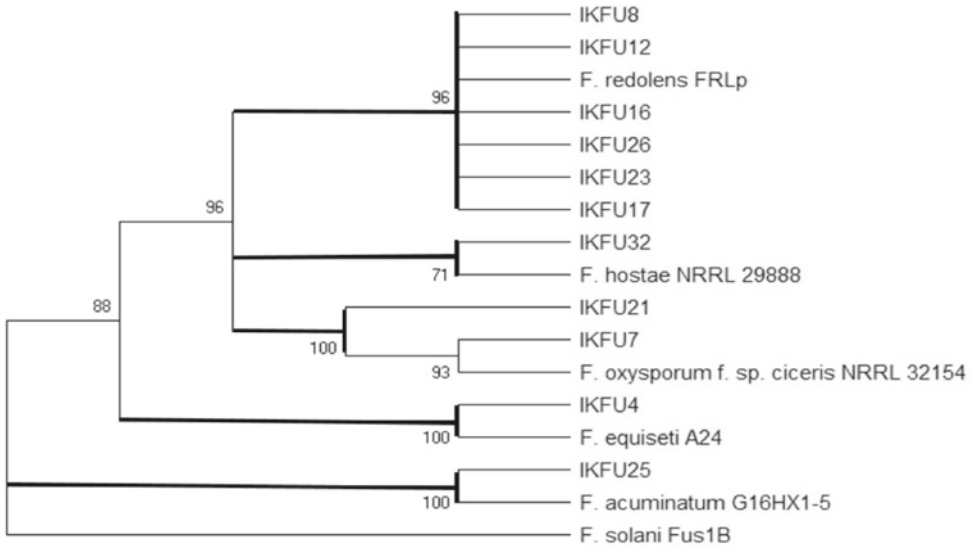

Fig. 3. A maximum parsimony consensus phylogeny using MEGA X with 1000 bootstrap replications inferred from concatenated DNA sequences of *tef1-α* and *tub-2* genes of *Fusarium* spp. isolates obtained from chickpea wilt infected plants and five reference isolates (FRLp, NRRL29888, NRRL32154, A24 and G16HX1-5) obtained from GenBank. *Fusarium solani* (Fus 1B) sequence obtained from GenBank was treated as the outgroup

was well supported with a high bootstrap value (100% MP). The isolates of this group were isolated from the upper stem part of chickpeas with symptoms of yellowing and wilting and vascular discoloration. These isolates were identified as *F. oxysporum* based on morphological characteristics. In the fourth group, the IKFU4 isolate was completely related to *F. equiseti* strain A24 and supported with a high bootstrap value (100% bootstrap MP). In the fifth group, a strong relationship (100% MP) was established between *F. acuminatum* G16HX1 obtained from GenBank and the IKFU25 isolate. Each of the fourth and fifth groups consisted of only a single isolate (Fig. 3).

Pathogenicity characterization

ANOVA test results revealed disease variability among *Fusarium* spp. isolates on chickpea cultivars. Average disease incidence of *Fusarium* spp. isolates varied within 28.00–100.00% and 52.67–100.00% on cvs. JG–62 and Bivenij, respectively. Three *Foc* isolates (IKFU21, IKFU6 and IKFU7) and one *F. redolens* isolate (IKFU12) showed the highest disease incidence (100%) on cvs. JG–62 and Bivenij. The lowest disease incidence belonged to *F. equiseti* IKFU11 and IKFU4, *F. hostae* IKFU32, and *F. acuminatum* IKFU25 and IKFU18 isolates. Other isolates showed moderate pathogenicity on both cultivars. Both cultivars showed about 100% of disease incidence at 50 to 60 days after sowing. All *F. redolens* isolates were pathogenic on the two chickpea cultivars (Table 3).

Symptoms of *F. redolens* and *Foc* were similar on aerial parts of the chickpea cultivars and included yellowing and wilting of aerial plant parts. Yellowing was first observed in the lower parts of the chickpea plants about 2 weeks after sowing and then gradually expanded to the top of the plants (Fig. 4A, E). About 40 days after sowing, the symptoms of yellowing were intensified. All chickpea plants died at 60 days after planting as a result of being infected by highly virulent isolates. No signs of vascular discoloration were observed in longitudinal or transvers sections of stems in plants affected by *F. redolens* isolates. Instead, brown to black necrosis was observed on the tap-root and crown (Fig. 4D).

In the case of *Foc*, longitudinal and transverse sections showed clear signs of discoloration of the vascular tissues (Fig. 4B, C, F). Symptoms observed under greenhouse conditions were similar to the field symptoms. The pathogens were re-isolated from diseased plants and untreated control plants remained healthy. In the case of *F. solani,* yellowing symptoms were first evident in upper parts of the chickpea plants and progressed downward gradually (data not shown). In this case, the roots showed extensive black root rot symptoms.

Discussion

The morphology-based identification of *Fusarium* spp. relies on a limited number of taxonomic characters with minor differences in morphology and varying importance in different *Fusarium* spp. (Lesli *et al.* 2001; Jiménez-Fernández 2011). Thus, molecular techniques should be used to overcome the faults of morphological characteristics. To meet this requirement, this study represents a comprehensive characterization of *Fusarium* spp. associated with Fusarium wilt of chickpea based on pathogenicity, morphological and molecular identification, and provides the phylogenetic analysis of concatenation of translation elongation factor 1-α (*EF-1*α) and *β-tubulin* gene regions on a set of *Fusarium* spp. isolates. The results of a phylogenetic tree were in agreement with morphological and species specific-PCR of FOC-PCR and FR-PCR protocols developed by Jiménez-Gasco and Jiménez-Díaz (2003) and Bogale *et al.* (2007), respectively. Based on phylogenetic analysis, the isolates belonged to five different species. *Fusarium oxysporum* f. sp. *ciceris* and *F. redolens* had the highest isolation frequency across all the sampling areas. *Fusarium hostae*, *F. equiseti* and *F. acuminatum* were minor isolates. The results confirmed the usefulness of molecular identification of *Fusarium* spp. based on the two gene loci studied. The competence and power of multigene phylogeny to resolve the phylogeny of species have been well described in a review article by Taylor *et al.* (2001).

In the present study, the isolates of *F. oxysporum* f. sp. *ciceris* formed a distinct clade that highly supported previous reports (Gams *et al.* 1999; Baayen *et al.* 2001; Geiser *et al.* 2001) on the *F. oxysporum* complex as a monophyletic group independent from *F. redolens*. The tree performed from combined *EF1-α* and *β-tubulin* sequence data showed a distinct clade among *F. redolens* isolates. Among them, *F. redolens* isolates from different geographical areas were scattered and no correlation was found between the geographical distribution and pathogenicity of isolates. As reported by Geiser *et al.* (2001) and Baayen *et al.* (2001), the phylogenetic analysis of sequences of *EF1-α* in combination with *β-tubulin* gene regions showed that *F. redolens* and *F. hostae* formed a separate monophyletic lineage.

Fusarium oxysporum f. sp. *ciceris* and *F. redolens* were the most abundant species associated with Fusarium wilt of chickpea and had a wide distribution in all the areas studied. It was found that the isolates of both species were pathogenic on chickpea and produced a similar symptom on aerial plant parts of chickpea cultivars. However, only *F. oxysporum* f. sp. *ciceris* isolates caused darkening of the vascular tissues in this

Table 3. Molecular identification of *Fusarium* spp. isolates and their pathogenicity test on two highly susceptible chickpea cultivars (Bivenij and JG-62); data represent mean ± standard deviation (SD) (n = 3)

Isolate code*	Species specific primers**			*EF1-α* and *β-tubulin****	Disease intensity index***** (*DII*)			
	FoF1/FoR1	Foc012f/ Foc012r	Redolens-F/ Redolens-R		Bivenij		JG-62	
IKFU1	+	+	–	Foc	98.00 ± 2.00	ab	97.67 ± 2.52	abc
IKFU2	+	+	–	ns	97.00 ± 1.73	abc	96.33 ± 2.89	abcd
IKFU3	–	–	+	Fr	87.67 ± 3.75	fg	71.67 ± 2.52	k
IKFU4	–	–	–	Fe	56.33 ± 2.08	k	31.67 ± 2.52	m
IKFU5	+	+	–	Foc	93.67 ± 3.21	bcd	90.00 ± 2.00	fg
IKFU6	+	+	–	ns	100.00 ± 0.00	a	100.00 ± 0.00	a
IKFU7	+	+	–	ns	100.00 ± 0.00	a	100.00 ± 0.00	a
IKFU8	–	–	+	Fr	97.00 ± 3.60	abc	96.00 ± 2.65	abcd
IKFU9	+	+	–	Foc	77.33 ± 2.08	h	85.67 ± 3.05	hi
IKFU10	+	+	–	ns	65.67 ± 2.52	j	80.67 ± 3.79	j
IKFU11	–	–	–	Fe	52.67 ± 3.05	kl	28.00 ± 2.00	m
IKFU12	–	–	+	Fr	100.00 ± 0.00	a	100.00 ± 0.00	a
IKFU13	–	–	+	ns	72.33 ± 4.73	i	62.67 ± 3.05	l
IKFU14	+	+	–	ns	91.33 ± 4.04	def	98.67 ± 1.15	ab
IKFU15	+	+	–	ns	73.33 ± 3.05	hi	88.67 ± 2.65	gh
IKFU16	–	–	+	Fr	88.00 ± 2.00	efg	65.33 ± 3.79	ll
IKFU17	–	–	+	ns	88.67 ± 3.51	efg	72.33 ± 3.51	k
IKFU18	–	–	–	Fa	54.67 ± 3.05	k	32.00 ± 2.65	m
IKFU19	+	+	–	Foc	86.67 ± 4.04	fg	97.67 ± 2.51	abc
IKFU20	+	+	–	ns	95.00 ± 3.00	bcd	97.33 ± 2.31	abc
IKFU21	+	+	–	ns	100.00 ± 0.00	a	100.00 ± 0.00	a
IKFU22	+	+	–	ns	94.67 ± 4.73	bcd	97.00 ± 2.65	abc
IKFU23	–	–	+	Fr	77.33 ± 2.08	h	91.33 ± 3.05	efg
IKFU24	+	+	–	ns	65.67 ± 4.51	j	81.67 ± 2.89	ij
IKFU25	–	–	–	Fa	53.33 ± 2.89	k	29.00 ± 3.60	m
IKFU26	–	–	+	ns	90.67 ± 3.51	def	89.33 ± 1.15	fgh
IKFU27	–	–	+	ns	84.67 ± 3.05	g	65.33 ± 3.05	l
IKFU28	+	+	–	Foc	92.67 ± 3.51	cde	92.33 ± 2.52	def
IKFU29	+	+	–	ns	85.33 ± 4.16	g	97.67 ± 2.52	abc
IKFU30	–	–	+	ns	71.00 ± 2.65	i	66.00 ± 3.00	l
IKFU31	–	–	+	ns	88.67 ± 3.51	efg	75.67 ± 2.52	k
IKFU32	–	–	–	Fh	56.00 ± 2.00	k	31.33 ± 3.51	m
IKFU33	+	+	–	ns	95.00 ± 3.00	bcd	94.33 ± 3.05	cde
IKFU34	+	+	–	ns	86.67 ± 3.05	fg	95.67 ± 2.52	bcd
IKFU35	–	–	+	ns	85.33 ± 2.52	g	95.67 ± 2.08	bcd
IKFU36	+	+	–	Foc	97.67 ± 2.52	ab	95.00 ± 3.60	bcde

*all isolates were obtained from either stem or lower stem tissues of chickpea plants

**PCR specific species primers: FoF1/FoR1, Foc012f/Foc012r and Redolens-F/Redolens-R developed by Mishra *et al.* (2003), Jiménez-Gasco and Jiménez-Díaz (2003) and Bogale *et al.* (2007), respectively, to confirm identification of *Fusarium oxysporum*, *F. oxysporum* f. sp. *ciceris* and *F. redolens* isolates; + amplicon was detected; – no amplicon was detected

****EF-1α* – translation elongation factor 1-α and *β-tubulin* gene regions; ns – not sequenced; Foc – *F. oxysporum* f. sp. *ciceris*; Fr – *F. redolens*; Fe – *F. equiseti*; Fa – *F. acuminatum*; Fh – *F. hostae*

****disease intensity index; comparison of means was carried out using the Least Significant Difference (LSD) at the level of $p \leq 0.05$

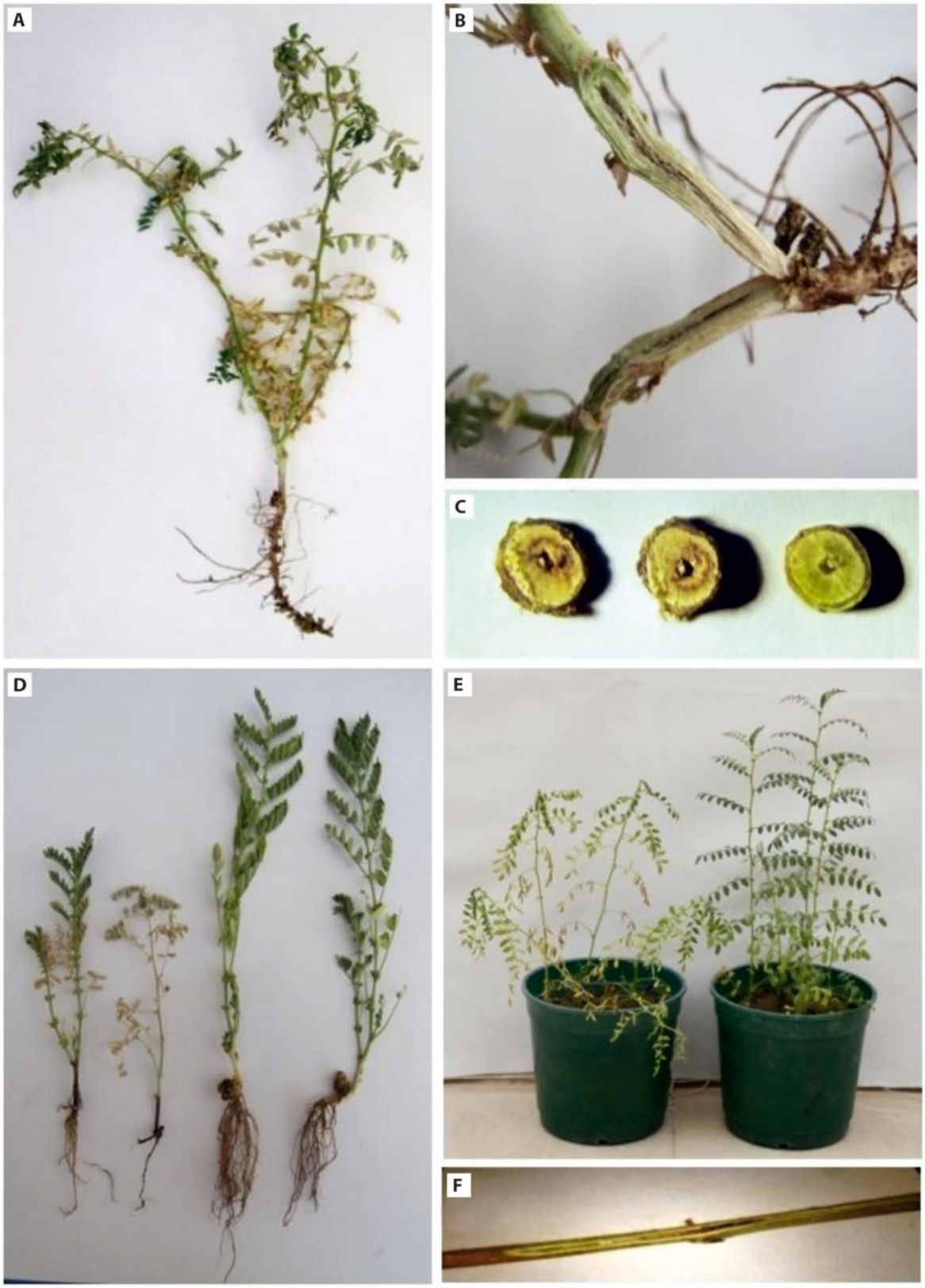

Fig. 4. Symptoms of wilt disease on a chickpea plant incited by *Fusarium oxysporum* f. sp. *ciceris* under field conditions (A–C) and pathogenicity test (E–F): inoculated (left) compared to non-inoculated control (right) (E); vascular tissue discoloration in longitudinal section (B, F) and transverse section (C). Symptoms of wilt disease on chickpea seedlings incited by *F. redolens* in pathogenicity test (D: infected – left, and healthy control – right)

study. *F. oxysporum* f. sp. *ciceris* isolates were highly aggressive on two chickpea cultivars of Bivenij and JG-62 in all pathogenicity tests. Isolates of *F. redolens* also created severe symptoms with artificial inoculation. All *F. redolens* isolates were pathogenic on two chickpea cultivars of Bivenij and JG-62. For instance,

isolate IKFU12 of *F. redolens* was highly virulent on both cultivars which died 60 days after sowing. This suggests that *F. redolens* can be considered as a potentially aggressive pathogen in chickpea fields in western Iran. There were low pathogenicity and isolation frequency of *F. hostae*, *F. equiseti* and *F. acuminatum* on chickpea cultivars. These findings can facilitate the development of successful chickpea wilt management programs in these areas based on accurate identification of chickpea wilt pathogens.

According to the overall results of this study, a disease complex caused by more than one pathogen species can be responsible for *Fusarium* wilt of chickpea in western regions of Iran. Based on the frequency of isolates, *Foc* appears to be the primary cause of chickpea Fusarium wilt disease in western Iran. Therefore, it should continue to be the first consideration of disease management. The occurrence of *Foc* in infected chickpea plants has been reported from Iran (Afshari Azad 1998; Younesi 2004; Mohammadi and Banihashemi 2005; Zokaee *et al.* 2012; Nourollahi *et al.* 2018; Chehri 2018; Younesi *et al.* 2019). Shokri *et al.* (2020) reported five races of *Foc* including R0, R1B/C, R1A, R6 and R5 with the highest abundance in western and northwestern areas of Iran.

The presence of *F. redolens* infecting chickpea plants in western regions of Iran has been reported recently (Saeedi and Jamali 2021). However, these authors isolated *F. redolens* from soil and roots of infected chickpeas with black root rot symptoms. In the present study, *F. redolens* isolates were isolated from the stem of chickpea plants with yellowing and wilting symptoms. Previous studies have shown that the disease symptoms including yellowing and necrosis in the lower parts of the plant were evident at about 2 weeks after sowing and gradually expanded to the top of the plant (Jiménez-Fernández *et al.* 2011). This is contrary to the current status of root rot diseases where yellowing is first observed in the upper part of the plant. Jiménez-Fernández *et al.* (2011) reported that all tested *F. redolens* isolates incited symptoms similar to previously reported yellowing for *F. oxysporum* f. sp. *ciceris*, but without vascular tissue discoloration. Black root rot is a local disease and distinguishing between this disease and systemic diseases like wilting is important from a plant pathological viewpoint. Therefore, the association of *F. redolens* with symptoms of yellowing and wilting on chickpeas in Iran was reported for the first time. Futhermore, to the best of our knowledge, the association of *F. hostae* with yellowing and wilting disease of chickpea appears to have been reported for the first time. Phylogenetically, *F. hostae* is most closely related to *F. redolens* from which it can be separated by differnces in the sequence of the *β-tubulin* and translation elongation factor *1-α* genes (Leslie and Summerell 2006). Together with *F. redolens*, *F. hostae* forms a clade that is distinct and intermediate between those for the *F. oxysporum* and *G. fujikuroi* species complexes (Baayen *et al.* 2001; Leslie and Summerell 2006). This fungus is similar to *F. redolens* Wollenw., as evidenced by shared characteristics in morphology and DNA sequence (Geiser *et al.* 2001).

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