



Morphology, pathogenicity and molecular identification of some *Fusarium* species within the *Gibberella fujikuroi* species complex from wheat in Syria

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Abstract

Fusarium is a worldwide distributed fungal genus and includes different phytopathogenic species which are able to infect cereals among other crops. This study was carried out to identify and characterize the isolates of *Fusarium* spp. in the *Gibberella fujikuroi* species complex (Liseola section) through morphological and molecular approaches. A total of 105 isolates of *Fusarium* spp. were recovered from crown and roots of wheat plants, showing typical symptoms, collected from four Syrian provinces during 2017-2018. Seventeen isolates in the *G. fujikuroi* species complex were identified as *F. proliferatum*, *F. verticillioides* and *F. andiyazi* based on morphological characteristics, and sequencing a partial translation elongation factor 1-alpha (TEF1- α) gene. The pathogenicity test showed that all recovered isolates of these three species were pathogenic, with low to moderate severity, on durum wheat seedlings under laboratory conditions. To our knowledge, this is the first report of *F. andiyazi* causing crown and root rot on wheat.

Key words – crown rot – *first report* – root rot – TEF1- α

Introduction

Wheat is the main cereal crop in all parts of the world, where bread is the staple food (Dib & Soussi 2004). The *Gibberella fujikuroi* species complex includes an assemblage of *Fusarium* species with similar and overlapping morphological traits that complicates their differentiation (Kvas et al. 2009). Diseases caused by *Fusarium* species in this species complex are among the most common diseases reported on agricultural crops worldwide (Hsuan et al. 2011). Species such as *F. verticillioides*, *F. sacchari*, *F. subglutinans*, *F. proliferatum* and *F. fujikuroi* have been implicated in diseases of agricultural crops including rice, sugarcane, maize and wheat (Marasas et al. 2006, Siti Nordahliawate et al. 2008, Mohammadi et al. 2016). They also produce a remarkable wide range of secondary metabolites or mycotoxins that have a negative effect on human and animal health (Leslie & Summerell 2006, Wolny-koładka 2014). In Syria, some species of *Fusarium* in the *Gibberella fujikuroi* species complex such as *F. proliferatum* and *F. verticillioides* have been implicated in head blight, crown and root rot of wheat (El-Khalifeh et al. 2009, Al-Chaabi et al. 2015, 2018).

Morphological identification of plant pathogenic fungi especially *Fusarium* is the first and the most difficult step in the identification process. There are limits on the use of morphological characters for the identification of species in the *G. fujikuroi* species complex as species (*F. proliferatum* and *F. fujikuroi*, *F. verticillioides* and *F. andiyazi*) have very similar morphology. Although morphological observations may not suffice for complete identification, a great deal of information is usually obtained on the culture at this stage (Rahjoo et al. 2008), and still play an important role in sorting isolates into groups before applying other methods of identification (Leslie & Summerell 2006).

Several studies proved that molecular approaches could support morphological diagnostics by providing a rapid and reliable assay for routine identification of *Fusarium* spp. (Bluhm et al. 2002, Abedi-Tizaki & Sabbagh 2012, Carvalhais et al. 2019, Minati & Ameen 2019, Hafez et al. 2020). Polymerase chain reaction (PCR)-based techniques are considered a powerful diagnostic method for the identification of *Fusarium* and other fungal species. The techniques serve as confirmation step to morphological characterization and can be used for direct identification (Spanic et al. 2010). Some researchers have used species-specific PCR assay to identify some *Fusarium* species such as *F. proliferatum*, *F. subglutinans* and *F. verticillioides* (Mulè et al. 2004). Other commonly used PCR approach for *Fusarium* identification are the amplification of the second largest subunit of RNA polymerase II (RPB2) and the translation elongation factor 1-alpha (TEF1- α) genes (Šišić et al. 2018). TEF1- α gene is commonly used for diagnosis and sequences for this gene are available through GenBank and through the FUSARIUM-ID databases (Geiser et al. 2004).

The main objectives of this study are to (i) identify some species within the *G. fujikuroi* species complex, isolated from root and crown regions of winter wheat in Syria, using morphological and molecular methods, and (ii) test their pathogenicity on wheat seedlings.

Materials & Methods

Sample collection

Infected wheat plants were collected from different regions of four Syrian provinces [Tartous, Latakia, Hama (Al-Ghab plain), and Sweida] (Fig. 1), during 2017–2018. Samples were put in paper bags, and transported to the laboratory. The crown, root, and stem base tissues of each plant were rinsed with tap water to remove soil particles and examined for lesions.

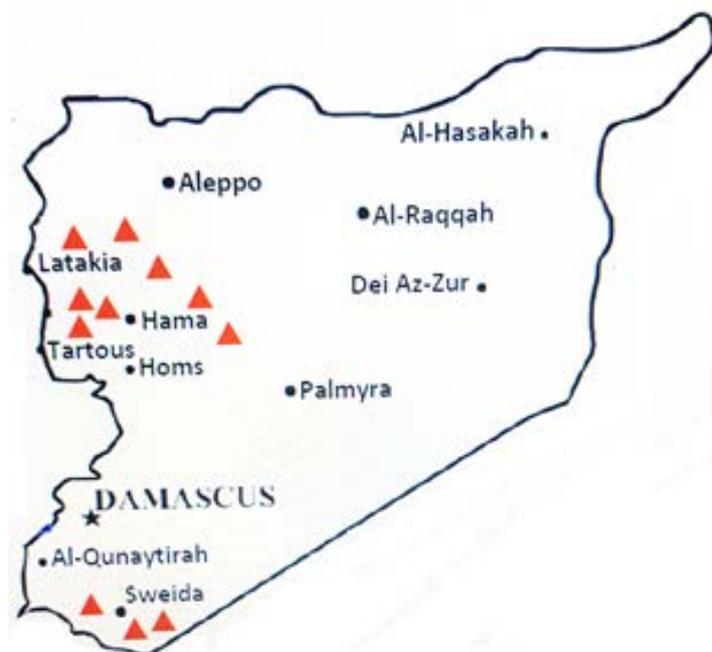


Fig. 1 – Map of Syria Provinces where *Fusarium* isolates were collected

Isolation of *Fusarium* spp.

Sections (3 cm) of symptomatic tissues were surface-sterilized with 1% sodium hypochlorite NaOCl for 3 min, rinsed with sterile distilled water, and air dried on sterilized filter paper in a laminar flow. Dried sections were cut into 1 cm in length and placed on potato dextrose agar (PDA) amended with 0.3 g L⁻¹ streptomycin and neomy cin sulphate, and incubated at 22±1°C in the dark for 7 days. *Fusarium*-like colonies were purified using the single spore isolation method (Nelson et al. 1983, Burgess et al. 1994). Single-spore cultures were grown on PDA in the dark at 25°C and 30°C for 72 h to study the growth rate as described by Burgess et al. (1994). Two media were used for the identification study: Potato Dextrose Agar (PDA) to study growth rate and the cultural appearances (colony colour and pigmentations), and Carnation Leaf Agar (CLA) to investigate microscopic characteristics (Burgess et al. 1994, Leslie & Summerell 2006).

DNA extraction

Fungal isolates were grown on PDA medium and incubated for 3–7 days at 25°C. The mycelium was harvested, transferred to a micro-centrifuge tube and suspended in 400µl extraction buffer (1.4 M sodium chloride, 0.1M Tris HCl, pH = 8, 20 mM EDTA Ethylenediaminetetraacetic acid, 2% CTAB Cetyltrimethylammonium bromide, 1% PVP polyvinyl pyrrolidone, 1% β-mercaptoethanol). Lysis of the mycelium was achieved by the addition of acid washed 0.4-0.6 mm diameter glass beads and 400µl phenol/chloroform/iso-amyl alcohol (Phe/Chl/IAA) (25:24:1). The mixture was vortexed at high speed for 10 min, centrifuged at 14000 rpm for 5 min, and 500 µl of supernatant were transferred to a new micro-centrifuge tube. 1000 µl of ice-cold ethanol 100% were added, mixed gently, incubated at -20°C for 1 h and centrifuged at 12000 rpm for 10 min to pellet the DNA. Supernatant was decanted, and DNA pellet was washed with 1000 µl of 70% ethanol. DNA pellet was air dried and dissolved in 50-75 µl TE buffer (10mMTris-HCl pH 8.0, 0.1 mM EDTA). 2µl RNase was added to DNA samples, mixed and incubated at 37°C for 45 min.

The DNA concentration was measured using NanoDrop Spectrophotometer (Thermo Scientific), and was adjusted to 100 ng/µL. DNA was then used as template for subsequent amplification using PCR.

Molecular identification based on partial *TEF1-α* gene sequence

Partial translation elongation factor 1-alpha (*TEF1-α*) gene sequence was amplified using primers ef1 (ATGGGTAAGGAGGACAAGAC) and ef2 (GGAAGTACCAGTGATCATGTT) (O'Donnell et al. 1998). The *TEF* partial sequence region was amplified in a 25 µl reaction mixture containing 12.5 µl *DreamTaq Green* PCR Master Mix (2X) (Thermo Scientific), 7.5 µl RNase-free water, 1.5 µl each of both forward and reverse primers (10 µM) and 2 µl of DNA. One isolate of each *Fusarium* species was selected for species confirmation. The PCR conditions were as follows: pre-denaturation at 95°C for 3 min; 10 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 2 min and final extension at 72°C for 10 min (Geiser et al. 2004).

Amplified products were analyzed using 1% agarose gel electrophoresis, and visualized under a UV transilluminator. Amplified samples showing discrete bands were subjected to automated DNA sequencing. Sequences were aligned using CLUSTAL X program (Thompson et al. 1997) to investigate genetic variation.

Pathogenicity test

Fusarium isolates were grown on PDA to prepare plugs for inoculum in the pathogenicity test. Experiments were conducted on a susceptible durum wheat cultivar Doma1. Glass tubes (2.5 cm in diameter × 20 cm in height) were filled with sterile soil mix [sand, soil, and organic matter (50:40:10 v/v/v) respectively] up to 5 cm below the top of the tube. A 1-cm diameter PDA agar plug from the periphery of a 7-day-old colony was placed on the soil surface in the tube. Seeds were germinated in Petri dishes for 4 days before being planted. A single germinated seed was placed on the agar plug and covered with soil mix. An agar plug with no fungus was used for the

control treatment. Seven *Fusarium* isolates were tested for their pathogenicity, and each fungal isolate represented a single treatment. Each treatment was replicated five times (each tube represented one replicate), and treatments were arranged in a randomized complete block design. The pathogenicity of 5 isolates of *F. culmorum* and *F. pseudograminearum*, obtained in previous study, were also tested for comparison. Plants were grown in a growth chamber at a temperature of 25/15°C day/night, with 16 h of photoperiod (Mitter et al. 2006), and watered as needed. Five weeks after fungal inoculation, plant roots were washed and scored for the typical symptoms of browning on the crown and the main stem base using a 1-5 scale (1: 0-9%, 2: 10-29%, 3: 30-69%, 4: 70-89%, 5: 90-100%) (Wildermuth et al. 2001, Gebremariam et al. 2017). For each *Fusarium* species, re-isolation was carried out, and the re-isolated cultures were confirmed as the corresponding *Fusarium* species by comparing their morphology with known cultures of the species. One-way analysis of variance was carried out using SPSS15 statistical program at $P \leq 0.05$.

Results

Morphological characterization

A total of 105 isolates of *Fusarium* spp. were obtained. Seventeen of them were identified within the *G. fujikuroi* species complex: 13 isolates of *F. proliferatum* (FT17, FH37, FT38, FH44, FH45, FT46, FT47, FH50, FS51, FS57, FS64, FS78, FT79), two of *F. verticillioides* (FT52, FT31) and two of *Fusarium andiyazi* (FT67, FT81) based on their morphological characteristics (Burgess et al. 1994, Leslie & Summerell 2006).

F. proliferatum isolates produced white aerial mycelia with a violet pigmentation on PDA after 10 days (Fig. 2a, b). The colony diameter ranged from 3-3.5 cm at 25°C, and 2.5-3 cm at 30°C after incubation in the dark for 3 days. Macroconidia were rare, slender, almost straight, 3-5 septate, curved apical cell, and distinct foot-shaped basal cell (Fig. 2c, d). Microconidia were oval to obovoid in shape, no septa (Fig. 2e), formed in false heads (Fig. 2f) and/or in short or long chains (Fig. 2g). Conidiogenous cells were monophialide (Fig. 2h) and polyphialide (Fig. 2i). Chlamydospores were absent.

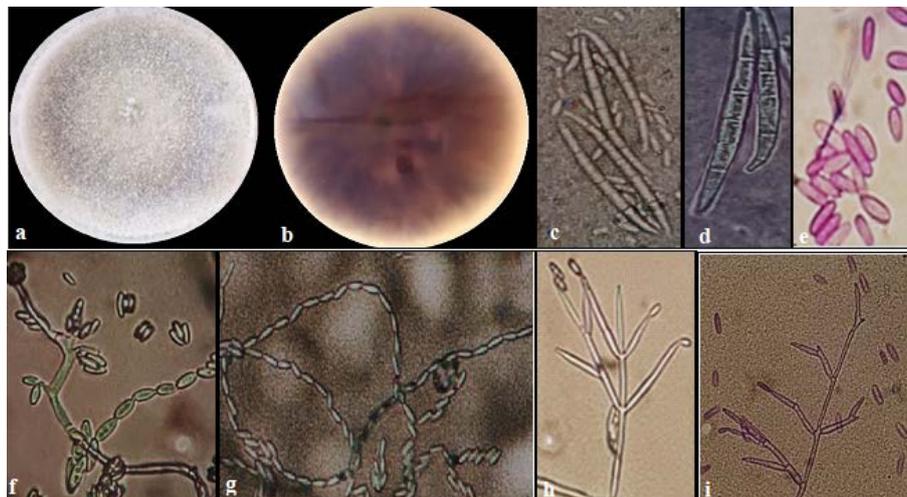


Fig. 2 – Morphological characteristics of *F. proliferatum* (isolate FT47). a upper surface of the colony. b lower surface of the colony. c 3-5-septate macroconidia. d macroconidia with distinct foot-shaped basal cell. e microconidia. f microconidia in false heads and in chains. g microconidia in long chains. h monophialide conidiogenesis. i polyphialide conidiogenesis.

F. verticillioides isolates produced white mycelia and developed violet pigments with age on PDA (Fig. 3a, b). The colony diameter ranged from 3-3.5 cm at 25°C, and 3.5-4 cm at 30°C after incubation in the dark for 3 days. Macroconidia were slender, slightly straight, 3-4 septate and

curved apical cell (Fig. 3c). Microconidia oval to obovoid in shape, no septa (Fig. 3d), formed in false heads (Fig. 3e) and/or in long chains (Fig. 3f) and they were formed from monophialides on the CLA (Fig. 3g). Chlamydo-spores were absent.

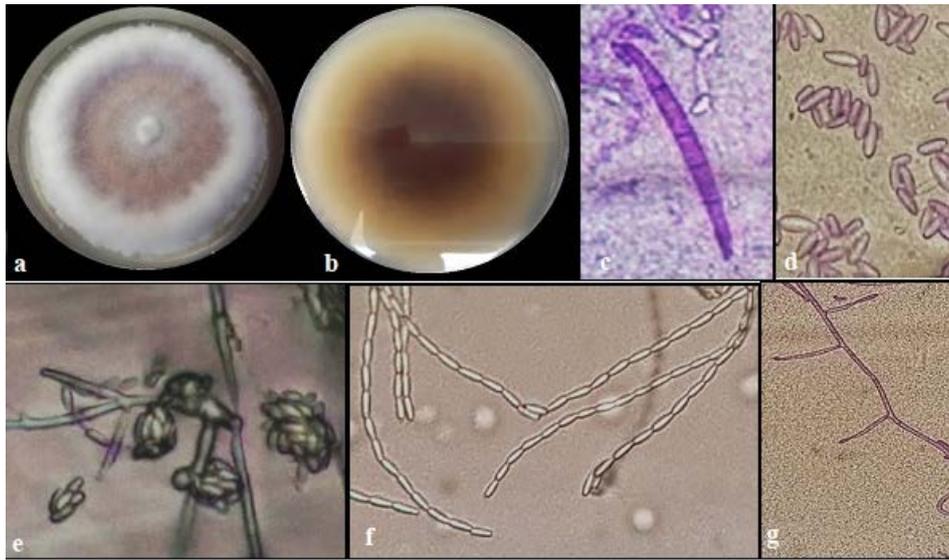


Fig. 3 – Morphological characteristics of *F. verticillioides* (isolate FT52). a upper surface of the colony. b lower surface of the colony. c 3-4-septate macroconidium. d microconidia. e microconidia in false heads. f microconidia in long chains. g monophialide conidiogenesis.

F. andiyazi isolates produced white aerial mycelia, with a violet pigmentation on PDA after 10 days (Fig. 4a, b). Colony diameter ranged from 3-3.2 cm at 25°C, and 2.7-3.5 cm at 30°C after incubation in the dark for 3 days. Macroconidia were slender, slightly straight, 3-5 septa, apical cell slightly curved, basal cell pedicellate (Fig. 4c). Microconidia abundant, clavate to ovoid, no septa (Fig. 4d), formed in false heads from monophialides (Fig. 4e), and in chains (Fig. 4f). Pseudo-chlamydo-spores are rarely present (Fig. 4g).

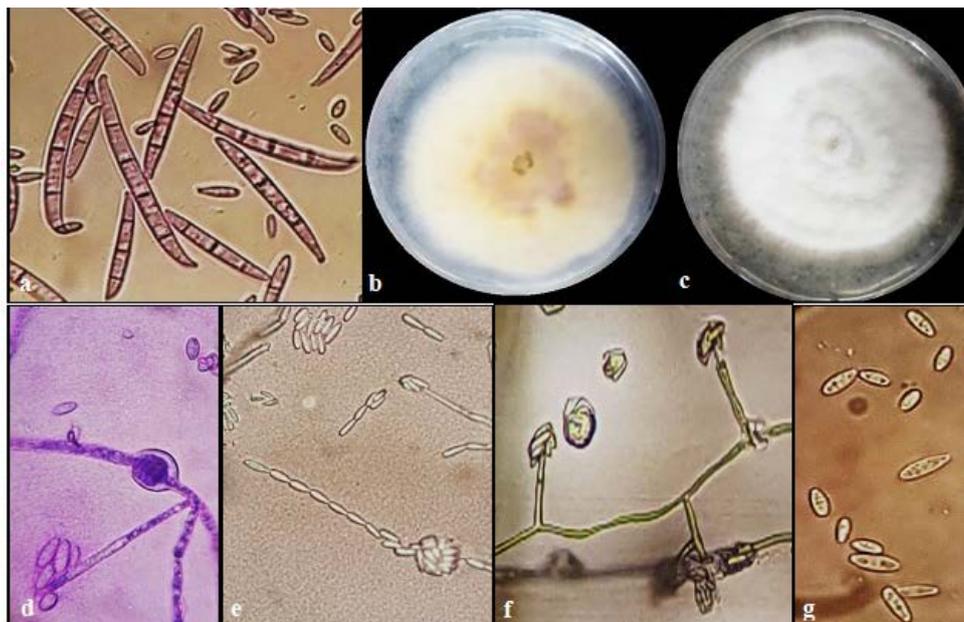


Fig. 4 – Morphological characteristics of *F. andiyazi* (isolate FT67). a upper surface of the colony. b lower surface of the colony. c 3-5-septate macroconidia. d microconidia. e false heads and monophialide conidiogenesis. f microconidia in chains. g pseudo-chlamydo-spore.

Molecular identification based on partial TEF1- α gene sequence

Amplified TEF1- α fragment size was ~700 bp in all tested isolates. A BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) confirmed species identification for FT47, FT52, and FT67 and showed 99.79%, 99.79% and 98.58% similarity with *F. proliferatum*, *F. verticillioides* and *F. andiyazi* respectively. Sequences were deposited in GenBank with the following accession numbers: MN865124 (FT47), MN807690 (FT52) and MN867480 (FT67). Sequences were aligned to investigate genetic variation (Fig. 5). A neighbor-joining phylogenetic tree was constructed using the Geneious tree builder software, where the consensus tree was based on the bootstrap resampling method of 1000 replications. This phylogenetic tree of partial TEF1- α gene shows close relationships between sequences of our isolates and reference sequences of *F. proliferatum*, *F. verticillioides* and *F. andiyazi* (Fig. 6). A confirmed Syrian isolate of *F. culmorum* (MN807691) was treated as the outgroup.

Pathogenicity test

A total of 12 isolates belonging to 5 *Fusarium* species (Table 1), were tested for their ability to cause crown rot on wheat seedlings. The tested species had different disease severity ranging from 0.6 – 3.8 depending on their virulence. *F. andiyazi*, *F. verticillioides* and *F. proliferatum* had low, low to intermediate and intermediate pathogenicity respectively (Fig. 7). The pathogenicity of the three species in the *G. fujikuroi* species complex was compared with those of *F. culmorum* and *F. pseudograminearum* that caused severe crown rot disease and death of wheat seedlings (Table 1).

Table 1 Mean disease severity index caused by 12 isolates of *Fusarium* spp. and scored 5 weeks after inoculating wheat seedlings of the cultivar Domal under laboratory conditions.

Fusarium species	Tested isolate ⁽¹⁾	Infected seedlings (%)	Infection severity of sub crown internode ^{(2) (3)}
<i>F. proliferatum</i>	FT17	100	1.2 cd
	FH44	80	1.4 d
	FS57	100	1.2 c d
<i>F. verticillioides</i>	FT52	80	0.8 bc
	FT31	80	1 bcd
<i>F. andiyazi</i>	FT67	60	0.6 b
	FT81	80	0.8 bc
<i>F. culmorum</i>	FH7	100	3.8 h
	FT27	100	2.4 e
	FS66	100	3.6 gh
<i>F. pseudograminearum</i>	FT63	100	2.8 ef
	FS87	100	3.2 fg
Control	-	0	0 a

(1) FT, FH and FS: isolates obtained from Tartous, Hama and Sweida provinces respectively

(2) Values followed by the same letter are not significantly different ($P > 0.05$) according to LSD tests. LSD at 5% = 0.513

(3) Scale = 0 – 5

Discussion

In this study, seventeen isolates were morphologically identified as *F. proliferatum*, *F. verticillioides* and *F. andiyazi*. In fact, distinguishing species within the *G. fujikuroi* species complex such as *F. proliferatum*, *F. verticillioides*, *F. andiyazi*, *F. fujikuroi* and *F. subglutinans* using morphological characters is a difficult procedure even for specialists (Summerell et al. 2003, Leslie & Summerell 2006, Hsuan et al. 2011). *F. proliferatum*, *F. verticillioides* and *F. andiyazi* have similar morphological characteristics like shape of the conidia, growth rate, colony morphology, and pigmentation on PDA (Burgess et al. 1994, Leslie & Summerell 2006). These species produce microconidia in chains or in false heads. The microconidia of *F. proliferatum* are

derived from polyphialide or/and monophialide conidiogenous cells, but the microconidia of *F. verticillioides* and *F. andiyazi* are only derived from monophialide conidiogenous cells (Burgess et al. 1994, Leslie & Summerell 2006). In the present study, it was very difficult to distinguish isolates of *F. verticillioides* and *F. andiyazi* because of the morphological similarity, and the only distinguishable character of *F. andiyazi* was the formation of very few pseudochlamydospores on PDA. In contrast, Venturini et al. (2017) reported that chlamydospores are absent in this species.

Due to the fact that using morphological characteristics can result in misidentification according to Rahjoo et al. (2008), molecular methods are usually needed for confirmation. The method of choice by several research groups is the characterization of *Fusarium* isolates based on the TEF1- α gene sequences (Gebremariam et al. 2017, Boutigny et al. 2019). Our results based on the partial DNA sequence of the TEF1- α region confirmed the morphological identification.

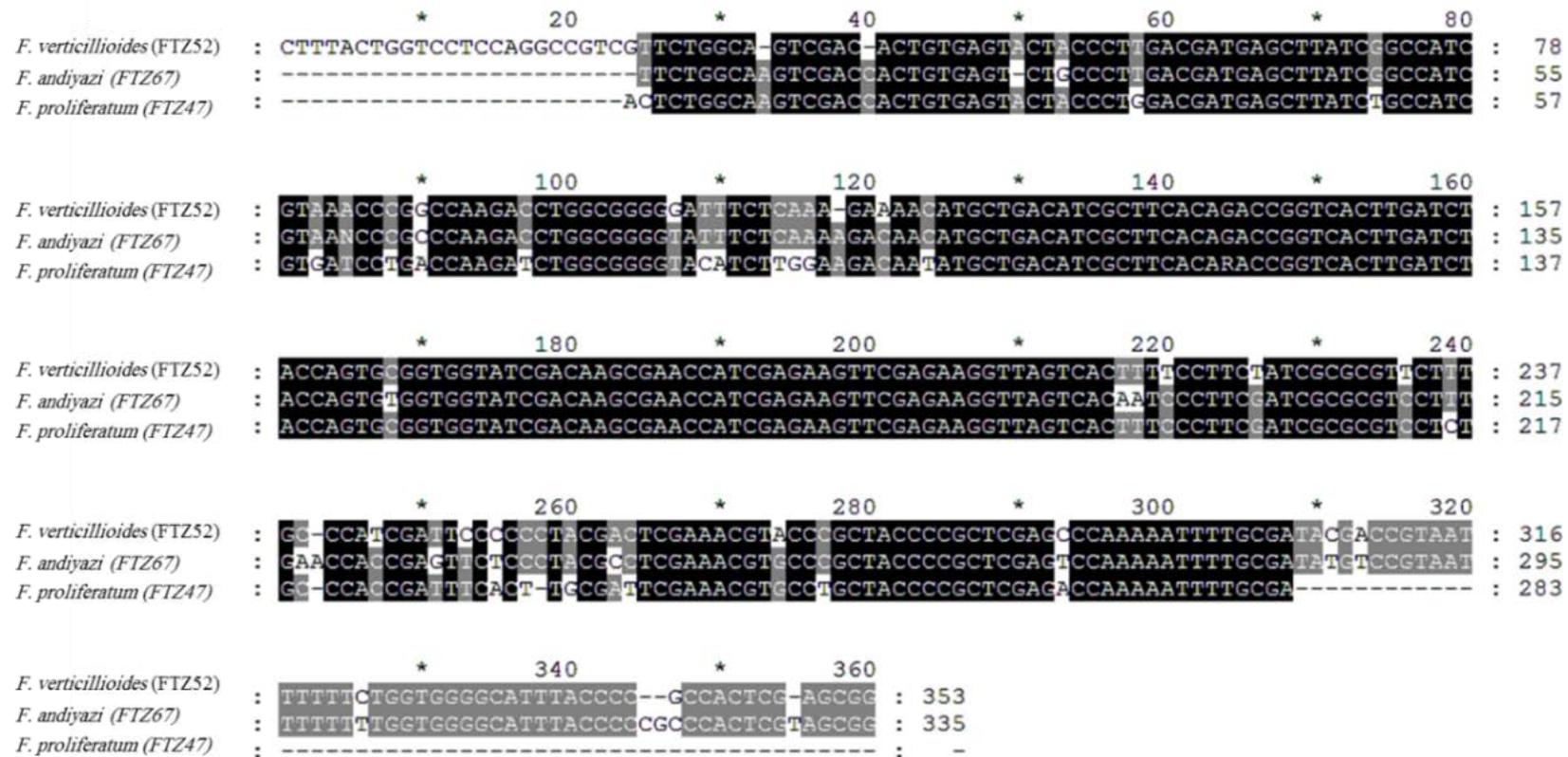


Fig. 5 – Alignment of partial TEF1- α gene sequences of three isolates of *F. proliferatum*, *F. verticillioides* and *F. andiyazi*

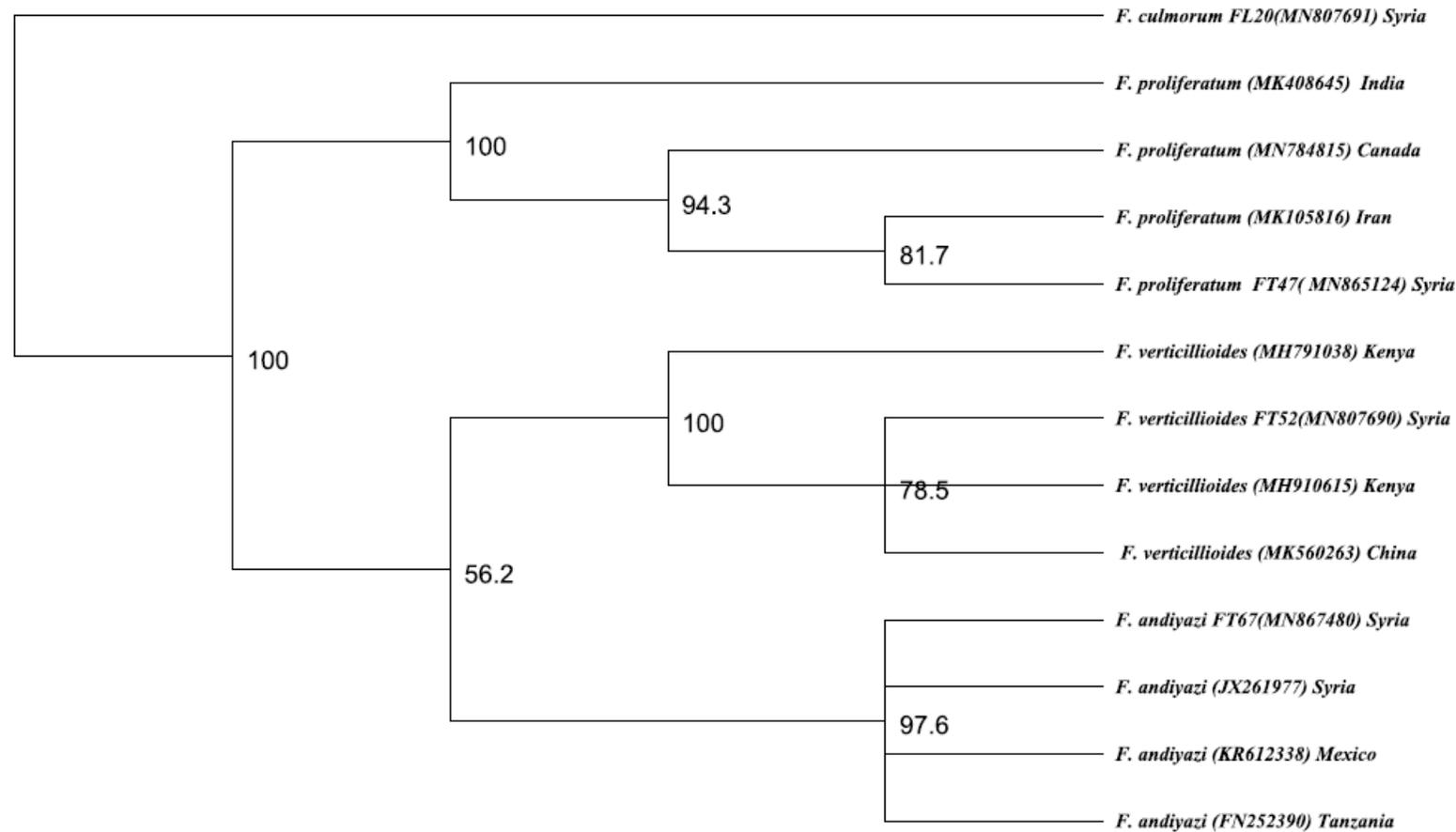


Fig. 6 – Neighbor-joining phylogenetic tree of partial TFE1- α gene showing relationships between sequences of Syria isolates and reference sequences of *F. proliferatum*, *F. verticillioides* and *F. andiyazi*. *F. culmorum* was treated as the outgroup.

All Isolates of *F. proliferatum*, *F. verticillioides* and *F. andiyazi* tested in this study caused disease on durum wheat seedlings under laboratory conditions, but they were less virulent compared with isolates of *F. culmorum* and *F. pseudograminearum* that caused a high disease severity. Al-Chaabi et al. (2015) reported *F. proliferatum* among the least virulent *Fusarium* species tested on durum wheat of variety ACSAD65 in Syria. Gebremariam et al. (2017) reported *F. proliferatum* as non-pathogenic species in Turkey. Although, *F. andiyazi* infected wheat seedlings under laboratory conditions, but it had relatively a low pathogenicity, and we can consider it as a weak pathogen. In fact, *F. andiyazi* is well known as a pathogen of maize (Leslie & Summerell 2006). It was first isolated and identified from Sorghum in Africa by Marasas et al. (2001), from rice grains

infected with Bakanae disease in Africa and Asia (Wulff et al. 2010), and from maize in Syria (Madania et al. 2013). In fact, some *Fusarium* species isolated from wheat have been also reported as saprophytic or weakly pathogenic. For example, *F. equiseti* has been reported as a wide spread and non-pathogenic *Fusarium* species (Summerell et al. 2003, Leslie & Summerell 2006, Gebremariam et al. 2017), while it was reported as pathogenic fungus on wheat, causing disease on the roots and crowns (Al-Chaabani et al. 2015, Fernandez & Chen 2005).

In summary, we have recovered three *Fusarium* spp. within the *F. fujikuroi* species complex from durum wheat in Syria. *F. proliferatum* and *F. verticillioides* are from the *F. fujikuroi* species complex and have been previously associated with crown and root rot of wheat. But, up to our knowledge, this study is the first report of *F. andiyazi* causing crown rot on wheat.



Fig. 7 – Durum wheat seedlings inoculated with *F. proliferatum* (a), *F. verticillioides* (b), *F. andiyazi* (c), non-inoculated control (d).

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