Morphological and Molecular Characterization of *Fusarium chlamydosporum*, *F. brachygibbosum* and *F. flocciferum* Associated with Crown and Root Rot of Wheat

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Abstract

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Fusarium is one of the most important genera of fungi, causing plant, animal, and human diseases and produces mycotoxins. A total of 105 isolates of *Fusarium* spp. were recovered from crowns and roots of wheat plants, showing typical symptoms, collected from four Syrian provinces during 2017-2018. In previous studies, seventeen *Fusarium* species associated with crown and root rot (FCR) on wheat were identified in Syria. However, the identity of some isolates has not been resolved. The current study was carried out to identify and characterize through morphological approaches and sequencing a partial translation elongation factor 1-alpha (*TEF1*-α) gene in three *Fusarium* species *F. chlamydosporum* Wollenweber & Reinking, *F. brachygibbosum* Padwick and *F. flocciferum* Corda associated with FCR, isolated and identified for the first time in Syria. The present study will provide detailed cultural and morphological characteristics of the three species, scarcely described in literature.

Keywords: First record, morphological descriptions, *TEF1*-α gene, Syria.

Introduction

Crown rot is considered among the most important wheat diseases caused by a complex of fungal pathogens including *Fusarium* spp., *Helminthosporium* spp., *Bipolaris sorokiniana* (Sacc.) Shoemaker, *Rhizoctonia* spp., *Alternaria* spp. *Microdochium nivale* (Fr.) Samuels & I. C. Hallett (El-Khalifeh *et al*., 2006; Smiley & Patterson, 1996). Common names of crown rot include dryland root rot, dryland foot rot, Fusarium foot rot, and Fusarium root rot (Smiley & Patterson, 1996). Fusarium crown rot (FCR) is caused by several species of *Fusarium*, particularly *F. culmorum* (W. G. Smith.) Sacc., *F. pseudograminearum* O'Donnell & Aoki, *F. graminearum* Schwabe and *F. avenaceum* (Fr.) Sacc. (Backhouse *et al*., 2004; Matny *et al*., 2019; Smiley & Patterson, 1996; Tunali *et al*., 2006).

Traditionally, *Fusarium* species have been identified based on their cultural and morphological characteristics, including colony pigmentation, the type of macro- and microconidia and shape and development of chlamydospores (Burgess *et al*., 1994; Leslie & Summerell, 2006), but this method is time consuming and difficult in some *Fusarium* species, therefore molecular methods such as DNA sequencing can be used for reliable identification and confirmation of *Fusarium* species.

The translation elongation factor 1- *α* (*TEF1-α*) gene, which encodes an essential part of the protein translation machinery, is commonly used for diagnosis, and its sequences are available in GenBank through the FUSARIUM-ID databases (Geiser *et al*., 2004; Madania *et al*., 2013). *TEF1*-α region is highly conserved and has been widely examined in intra- and inter-specific variation studies and phylogenetic analyses of a wide variety of eukaryotic groups, including fungi which belong the *Fusarium* genus (O'Donnell *et al*., 1998).

In previous studies (Zidan *et al*., 2020a, 2020b), seventeen *Fusarium* species associated with crown and root rot of wheat in Syria were identified. However, the identity of some isolates has not been resolved. The aim of this study was to identify three *Fusarium* species isolated from wheat with crown rot symptoms based on morphological and molecular characteristics.

Materials and methods

Sample collection

Infected wheat plants were collected from four Syrian provinces [Tartous, Latakia, Hama (Al-Ghab plain), and Sweida], during 2017–2018. Samples were placed in paper bags and transported to the laboratory. The roots were rinsed with tap water to remove soil particles and were examined for lesions.

Isolation of *Fusarium* spp.

Sections (3 cm) of symptomatic tissues were surface sterilized with 1% sodium hypochlorite 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 neomycin sulphate, and incubated at 22±1°C in the dark for 7 days. *Fusarium*-like colonies were purified using the single spore isolation method (Burgess *et al*., 1994). Two media were used for the identification study: potato dextrose agar (PDA) to study the cultural appearance (colony colour and pigmentations), and carnation leaf agar (CLA) to investigate microscopic characteristics (Burgess *et al*., 1994; Leslie & Summerell, 2006).

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DNA extraction

Fungal isolates were grown on PDA medium and incubated for 7 days at 25°C. The mycelium was harvested, transferred to a micro-centrifuge tube and suspended in 400 µl 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 pyrrolidon, 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 (10 mMTris-HCl pH 8.0, 0.1 mM EDTA). Two microliters of RNase were added to DNA samples, mixed and incubated at 37°C for 45 min.

The DNA concentration was measured using NanoDrop Spectrophotometer (Thermo Scientific), and adjusted to 100 ng/µL. DNA was then used as a 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 (GGAAGTACCAGT GATCATGTT) (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, 3 µl of forward and reverse primers (10 µM) and 2 µl of template DNA. One representative isolate of each *Fusarium* species was selected for species confirmation. The PCR conditions used 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 using1% agarose gel electrophoresis and visualized under a UV transilluminator. Amplified samples showing discrete bands were subjected to automated DNA sequencing.

Results

In previous studies, seventeen *Fusarium* species were identified from a total of 105 isolates of *Fusarium* spp. In the present study, three additional *Fusarium* species were identified: *F. chlamydosporum* based on its morphological characteristics and further confirmed by sequencing a partial (*TEF1*-α) gene, *F. brachygibbosum* and *F. flocciferum* based on sequencing a partial (*TEF1*-α) gene. Sequences of the identified isolates that represent three species were deposited in GenBank with the following accession numbers: MN883030 (FS89), MN883029 (FS88) and MN883031 (FS96), respectively.

Because of the scarcity of studies related to the morphological characteristics of these three species, the present study provides a detailed description of their cultural and morphological characteristics.

F. chlamydosporum: Isolates can be easily differentiated morphologically on PDA and CLA media. Colony surface was white and pink in the center and it became pink with aging and produced pink to red pigment in the agar (Figure 1a, 1b, 1c, 1d). Macroconidia were rare, slender, slightly straight, 3-4 septa, apical cells slightly curved, basal cells notched (Figure 1e). Microconidia were abundant, mainly 0-1 septa, single, straight or obovoid with appointed base (Figure 1F), they were produced from elaborate polyphialides that result in branching conidiophores with a tree-like appearance (Figure 1G, 1H). Chlamydospores were abundant, globose occurred in chains or in clusters, but more common as single or in pairs (Figure 1I, 1J, 1K). Based on these characteristics, the fungal isolate was identified as *F. chlamydosporum*.

F. brachygibbosum: Colony surface was white at first with abundant aerial mycelium, turning flat, pink with yellow hypha at the center. It produced pink to red pigment in the agar (Figure 2a, 2b, 2c, 2d). Macroconidia were slightly curved with four to five septa, the cells in the middle were slightly wide. The apical cells were with slightly sharp apexes, whereas the basal cells were slightly foot-shaped (Figure 2e, 2f, 2g). Microconidia were oval to cylindrical with 0 or 1 septa and often pointed at one end (Figure 2h). Conidiogenous cells were monophialides (Figure 2i, 2j). Spherical chlamydospores were terminal and intercalary, solitary or in clusters (Figure 2k, 2l, 2m).

F. flocciferum: Colony surface was pale rosy buff in the center, becoming pink and white towards margins. It produced red pigment in the agar (Figure 3a, 3b). Macroconidia were straight to curved with four to five septa (Figure 3c). Microconidia were variable in shape with 0 or 1 septa (Figure 3d). Conidiophores rather loosely branched with 2 - 3 terminal phialides for each branch (Figure 3e, 3f, 3g). Chlamydospores grew at the end of the hypha and were in chains (Figure 3h).

A neighbor-joining phylogenetic tree was constructed using the Geneious tree builder software, where the consensus tree was based on the bootstrap resembling method of 1000 replications (Figure 4). This phylogenetic tree of partial TEF1-α gene showed close relationships between sequences of our isolates and reference sequences of *F. chlamydosporum* (MN883030), *F. brachygibbosum* (MN883029) and *F. flocciferum* (MN883031). A confirmed Syrian isolate of *F. culmorum* (MN807691) was treated as the outgroup.

Discussion

Crown rot is a disease of worldwide distribution and importance. In wheat, this disease is caused by a complex of *Fusarium* species, causes significant yield losses in different regions worldwide. Crown rot has been reported in Syria (Al-Chaabi *et al*., 2015; Zidan *et al*., 2020a, b) and many other countries such as the USA (Smiley & Patterson,

1996), Canada (Fernandez *et al*., 2007), Australia (Backhouse *et al*., 2004; Akinsanmi *et al*., 2004), China (Zhang *et al*., 2015), Turkey (Bentley *et al*., 2006; Gebremariam *et al*., 2018), Iran (Darvishnia, 2013), and Iraq (Khalifah and Matny, 2013).

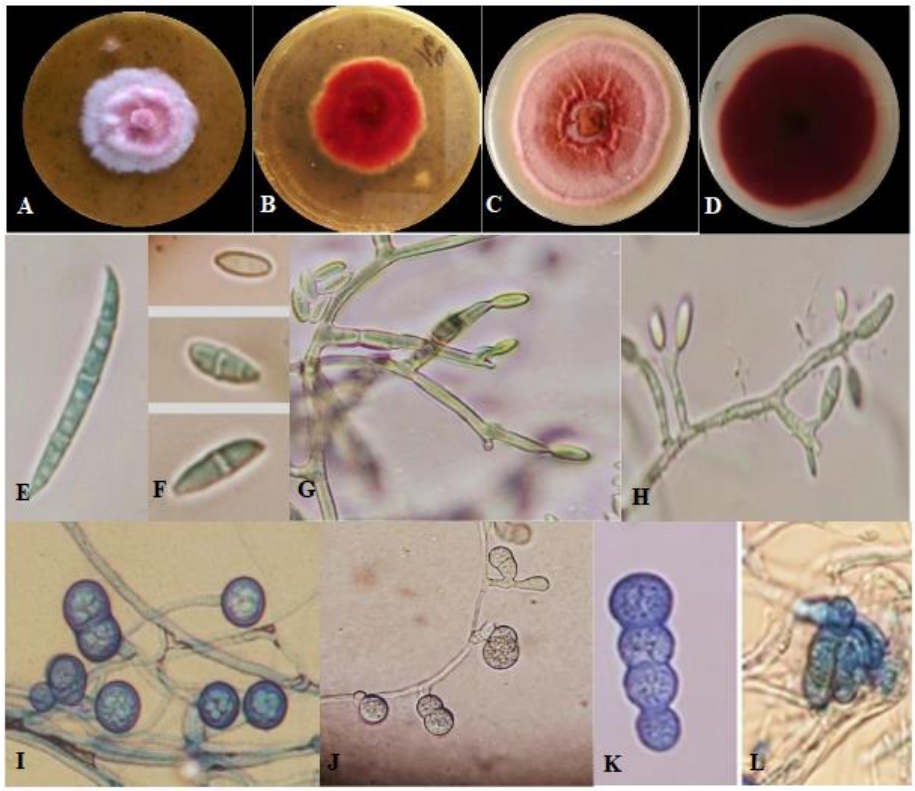

Figure 1. *Fusarium chlamydosporum* (FS89). A & B: 5 days old colony on PDA (A- surface, B-reverse), C & D: 10 days old colony on PDA (C- surface, D-reverse), E: macroconidia, F: microconidia, G & H: polyphialide conidiogenesis, I, J, K & L: chlamydospores.

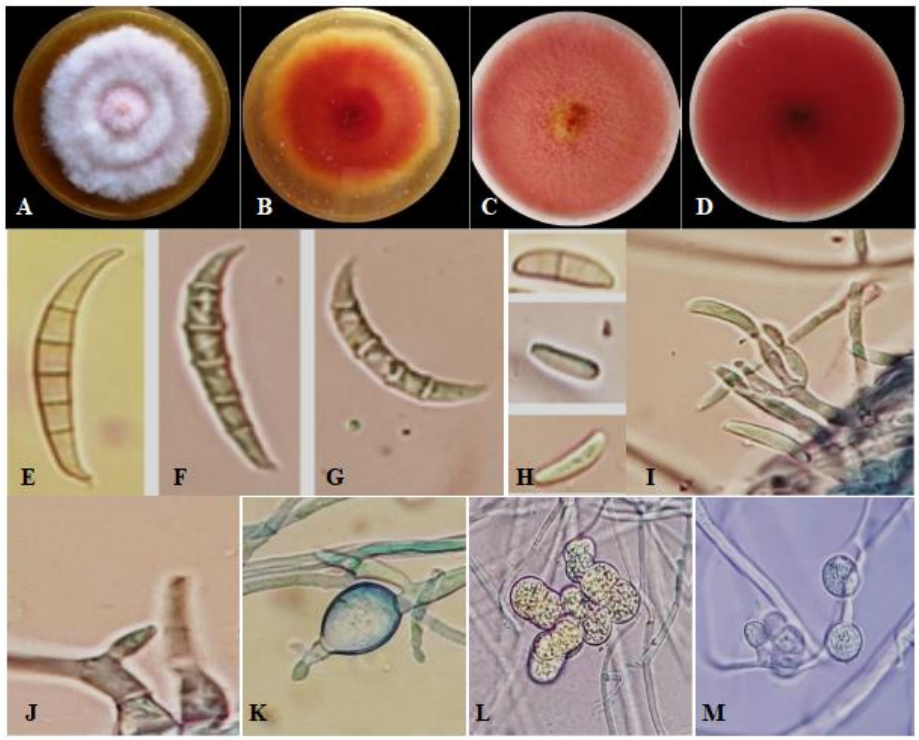

Figure 2. *Fusarium brachygibbosum* (FS88). A-B: 5 days old colony on PDA (A- surface, B-reverse), C–D: 14 days old colony on PDA (C- surface, d-reverse), E-F-G: macroconidia, H: microconidia, I-J: monophialide conidiogenesis, K-L-M: chlamydospores.

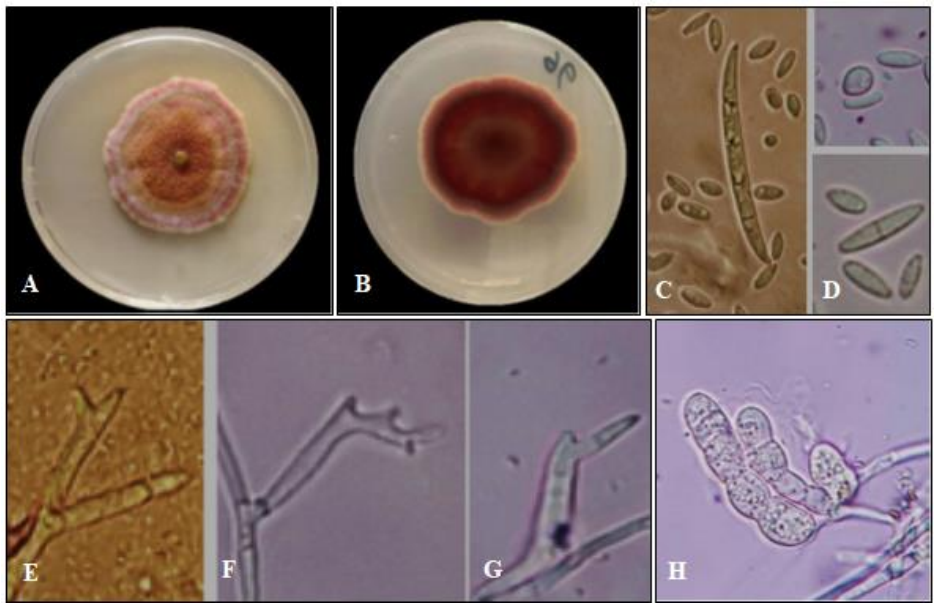

Figure 3. *Fusarium flocciferum* (FS96). A- B: 5 days old colony on PDA (A- surface, B-reverse), C: macroconidium and microconidia, D: microconidia, E-F-G: conidiophores with 2 - 3 terminal phialides to each branch, H: chlamydospores.

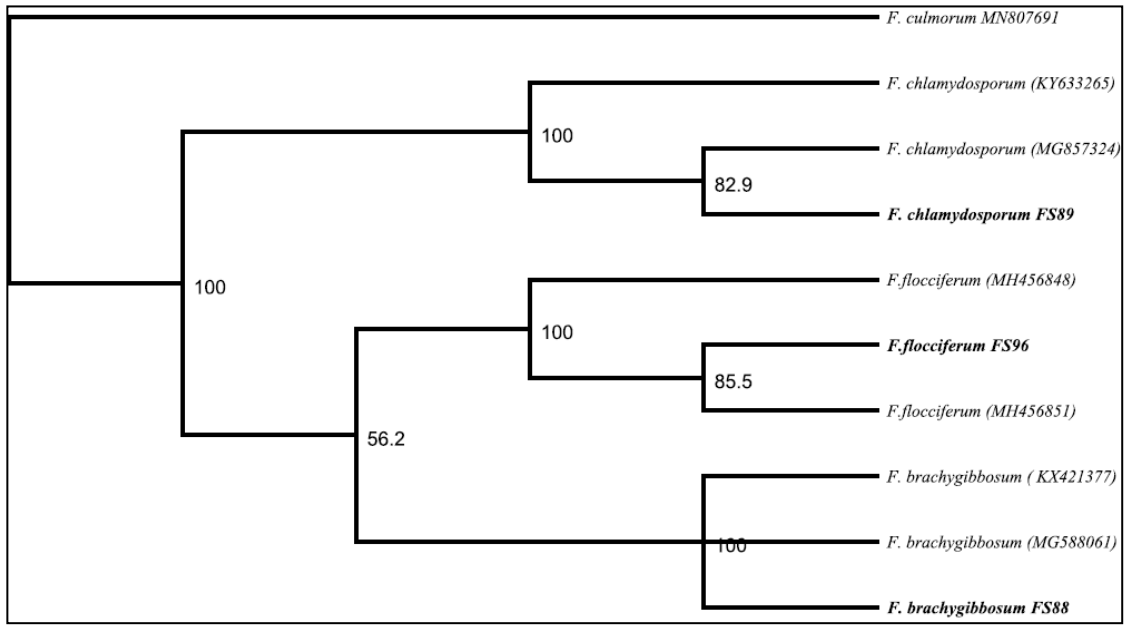

Figure 4. Neighbor-joining phylogenetic tree of partial TFE1-α gene showing relationships between sequences of Syrian isolates (FS89, FS96, FS88) and reference sequences of *F. chlamydosporum, F. brachygibbosum* and *F. flocciferum, with F. culmorum* (MN807691) treated as the outgroup.

Although there are many studies associated with crown rot disease in the world, few studies were conducted in Syria. One of them was done 15 years ago by Al-Chaabi *et al*. (2015). The findings of that study, based on morphological characteristics, indicated the presence of three *Fusarium* species *F. culmorum, F. proliferatum* and *F. equiseti* on wheat in six Syrian provinces (Aleppo, Daer Al-Zor, Al-Raqqa, Al-Hasakeh, Hama and Daraa).

In recent studies, *F. pseudograminearum, F. culmorum, F. sambucinum, F. compactum, F. acuminatum, F. solani, F. semitectum, F. pseudocircinatum, F. oxysporum, F. proliferatum, F. verticillioides, F. sporotrichioides, F. equiseti* associated with crown rot on wheat were isolated from four Syrian Provinces (Tartous, Latakia, Hama and Sweida) and identified based on morphological and molecular methods. A study in Syria

reported *F. andiyazi* for the first time in the world as a causative pathogen of crown and root rot on wheat (Zidan *et al*., 2020a). Another study reported the presence of *F. torulosum* for the first time in Syria (Zidan *et al*., 2020b).

Accurate identification of *Fusarium* species could be useful for designing novel and effective disease management strategies. A number of factors, including a lack of clear morphological characters for separating species, the high morphological similarity within some *Fusarium* species, together with variation and mutation in culture, can result in misidentification. To overcome these factors molecular identification is commonly applied (Demeke *et al*., 2005; Jurado *et al*., 2006; Niessen & Vogel, 1998; Mulé *et al*., 2004). The three *Fusarium* species obtained in this study have been isolated from wheat crowns and identified according to the morphological characteristics and by using *TEF1*-α gene sequencing. *TEF1*-α is a genetic marker considered optimal for identification of unknown *Fusarium* species (Geiser *et al*., 2004). The ef1/ef2 primers were used for molecular identification (O'Donnell *et al*., 1998).

Based on morphological characteristics that were stated by Leslie and Summerell (2006), the isolate (FS89) was identified as *F. chlamydosporum*. Sequencing a partial region of *TEF1*-α gene confirmed our morphological identification and a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed 99.03% similarity with *F. chlamydosporum* (GenBank accession No. MG857324).

Due to the high morphological similarity with *F. transvaalense*, FS88 was initially identified as *F. transvaalense* based on morphology according to Sandoval-Denis *et al*. (2018). Species identity was confirmed by sequencing part of the translation elongation factor 1- *α* (*TEF1-α*) gene. Depending on the analyses of the sequence in the NCBI GenBank databases, the isolate showed 99.37% similarity with *F. brachygibbosum* (GenBank accession No. MG588061).

Due to the inadequate available morphological information on *F. flocciferum*, it was not possible to identify the isolate FS96 based on morphological characteristics, so DNA sequencing of partial (*TEF1*-α) gene was used and compared with sequences in the NCBI GenBank database. The sequencing comparison showed high similarity (99.53%) with *F. flocciferum* (GenBank accession No. MH456851). For *Fusarium*, *TEF-1*-α gene is recommended for species identification as this gene occurs as a single copy and has a high level of sequence polymorphism among closely related species (Geiser *et al*., 2004).

Fusarium brachygibbosum has been isolated and identified as a pathogen in many plants such as wheat (Gebremariam *et al*., 2018), legumes (Tan *et al*., 2011), almond trees (Stack *et al*., 2017), maize (Shan *et al*., 2017), olive trees (Trabelsi *et al*., 2017) and sugar beet (Cao *et al*., 2018). *F. flocciferum* and *F. chlamydosporum* have been also identified as pathogens in many plants including wheat (Tunali *et al*., 2008; Gebremariam *et al*., 2018), but they were not isolated from any plant in Syria until this study.

According to most previous studies, *F. brachygibbosum* and *F. flocciferum* were identified through molecular techniques, and only a brief morphological description was given, so the present study described their cultural and morphological characteristics.

To our knowledge, this is the first report of a crown rot disease on wheat in Syria caused by *F. brachygibbosum*, *F. chlamydosporum* and *F. flocciferum*.

الملخص

زيدان، ليلى، دانا جودت ووليد نفاع. 2022. الخصائص المظهرية والجزيئية لثلاثة أنواع من فطر الفيوزاريوم مرافقة لعفن التاج والجذور في القمح. مجلة وقاية النبات العربية، 40(2): 175−181. https://doi.org/10.22268/AJPP-040.2.175181

يُعدّ الجنس *Fusarium* من أهم الأجناس الفطرية المسببة أمراضاً للنباتات والحيوانات والإنسان، إضافة لقدرته على إنتاج سموم فطرية mycotoxins. تمّ الحصول على 105 عزلات تتبع أنواعاً مختلفة من الجنس *Fusarium* من منطقة التاج والجذور لنباتات قمح أبدت أعراض إصابة نموذجية، جُمعت من أربع محافظات سورية خلال العامين 2017 و 2018. في دراسات سابقة، تمّ تعريف 17 نوعاً من الفيوزاريوم مرتبطة بعفن التاج والجذور على القمح في سورية؛ ومع ذلك، إنّ بعض العزلات لم يتمّ تعريفها بشكلٍ دقيق، وعليه، هدفت هذه الدراسة إلى تعريف وتوصيف ثلاثة أنواع Wollenweber & Reinking *F. chlamydosporum*، Padwick *F. brachygibbosum*، و Corda *F. flocciferum* مرافقة لعفن التاج والجذور على القمح، والتي تمّ عزلها وتعريفها لأوّل مرّة في سورية، وذلك بالاعتماد على الخصائص المظهرية، والتسلسل الجزيئي لمورثة عامل الاستطالة والترجمة *TEF1*-α. تقدم هذه الدراسة وصفاً مورفولوجياً دقيقاً لهذه الأنواع الثلاثة، وذلك نظراً لندرة الدراسات المرجعية السابقة المتعلقة بها.

كلمات مفتاحية: التسجيل الأول، وصف مورفولوجي، المورث *TEF1*-α، سورية.

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