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The First Record of *Fusarium incarnatum* as Wilting Agent on Tomato Plants in Iraq

Rasha Khalid Hussein AL-Masoudi [1*], Neamat J. AL Judy [2]
[1,2] Department of Biology , College of Science, University of Baghdad, Baghdad, Iraq.
*Corresponding Author.

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Abstract

Fusarium wilt is one of the most significant diseases that affects tomato plants and is caused by the fungus *Fusarium oxysporum*, whether in fields or greenhouses. The current research aimed to identify the *Fusarium incarnatum* (Desm.) Sacc. agent that causes *Fusarium* wilt on tomato plants for the first time in Iraq by morphological and molecular methods, in addition to detecting the phylogenetic distance and similarity of the Iraqi isolates to other isolates globally. In January of 2023, the wilt signs were apparent on wilted tomato plants in Al-Mahmoudiya fields in Iraq. The isolate was confirmed and molecularly diagnosed according to the internal transcribed spacer (ITS) region. The sequences of ITS region for the isolate have been presented to the database in the NCBI Gen Bank for the first time, and it obtained their accession number (OQ439282.1). *F.incarantum* was isolated and purified and identified by using morphological and microscopic features, the morphological features of *F.incarnatum* isolate were observed on PDA media and under a microscope revealed that macroconidia have curved shapes, three to five septate, and basal cells with a foot shape. The pathogenicity of the fungus *F.incarnatum* showed the fungus's ability to cause wilting disease in healthy tomato plants at 90.33%, this demonstrates that the *Fusarium* isolate can create strains that can attack the host continually. Additionally, the phylogenetic tree revealed the genetic relationship between the Iraq isolate and other global isolates and it showed the Iraq isolate (OQ439282.1) is in the same clade as the Turkey isolate, India isolates, and China isolate with similarity 100%, and this clade shares 99.60% similarity with another clade, including Thailand, Taiwan, Nigeria, South Korea, Pakistan, Japan, Egypt, USA, and Tunisia isolates. As far as we are aware, this is the first morphological and molecular recording of *F.incarantum* on an Iraqi tomato plants.

Keywords: *Fusarium incarnatum*, Iraqi isolate, Phylogenetic tree, Tomato Plant, Wilting agent.

1. Introduction

It is well known that *Fusarium* species can infect a wide variety of host plants with a wide range of diseases (1, 2). Soil-borne filamentous fungi have an important economic role since many of their species are responsible for vascular wilt diseases in ornamental and agricultural crops across the world. (3). The pathogen can attack the plant because this fungus can remain alive latently for prolonged periods in the soil in the form of spores (4). One of the crops of

the Solanaceae family, the tomato plant (*Solanum Lycopersicon* L.), is important due to its great nutritional value to humans (5). The popular fungus associated with tomato roots is *Fusarium oxysporum* f. sp. *lycopersici*, which is the causative factor of tomato wilt; yet, several additional species have been discovered from wilted tomato plants (6).

The *Fusarium* genus is quite diverse and has more than 1650 species that belong to it; some of these species are registered in the gene bank and others are not, but the number is still vulnerable to rise(7). *F.incarnatum* (Desm.) Sacc. is widespread in subtropical and temperate climates and affects crops such as sorghum, corn, and rice (8). Fumonisin and deoxynivalenol, two toxins produced by *F. incarnatum*, result in clinical symptoms such as diarrhea and vomiting (9). *Fusarium* species are commonly identified by their macroscopic and microscopic characteristics (10). Over the past 20 years, molecular technologies have been shown to have a great benefit in the detection of plant pathogens(11). It might be challenging to diagnose *Fusarium* at the species level only based on morphological features because of the genetic variation of the fungus (12).

PCR-based assays are the most popular way to diagnose and distinguish between species and sub-species and are regarded as a rapid procedure (13). Since the internal transcribed spacer (ITS) gene is an advantageous genetic area for research on the *Fusarium* species, it was employed in the *Fusarium* -ID database as an aid for identification (14). These sequence data have been extensively employed in the phylogeny and taxonomy of the species of *Fusarium* (15). Consequently, The research aims to identify the *F. incarnatum* agent that caused the wilt of *Fusarium* on tomato plants for the first time in Iraq.

2. Materials and Methods

2.1. Morphological study of the Fungus

In January of the year 2023, symptoms of wilt were observed on tomato plants in many fields in Iraq but *F. incaranatum* was isolated only from the fields in Al-Mahmoudiya in Iraq. Samples were collected from tomato plants that were exhibiting symptoms. Root and stem samples were cut off with a size of (5 mm) by using a sterilized blade into five pieces, it was taken from the infected plant tissues (16). Root and stem specimens were sterilized by submersion in a solution of sodium hypochlorite (1% free chlorine) for 2–5 min and washed fully with distilled water, after that, it was cultivated in Petri dishes with sterilized Potato Dextrose Agar (PDA) media, and the plates were incubating for 7 days at a temperature of at 25±2°C. In the purification of fungal growth on PDA medium, the colony shape was observed in PDA media. Fungal mycelium was microscopically examined on a glass slide under a microscope to describe the shape of macroconidia, microconidia, and chlamydospores to confirm the relationship with *Fusarium* through the morphological characteristics described in (17) .Many species of *Fusarium* were diagnosed, such as *F. oxysporum* and *F. proliferatum*, but *F.incarnatum* was recorded for the first time as a wilting agent in tomato plants, and its appearance rate was 70٪ while the frequency rate was 48٪.

2.2. Pathogenicity test

It was prepared with a mingle of peat moss together with sandy soil at a rate of 2: 1 and disinfected with a formalin. A 6% formalin concentration was added to the soil. The soil was then put into plastic bags, and a suitable volume was added of 45 ml of formalin for each 10 kilograms of the soil. The plastic bags had been properly sealed before being set aside for 5 days. After that, when the bags were opened, the soil was distributed in clean plastic pots., and it was exposed to the outside air for three days, to eliminate the formalin smell. The sterilized soil was dispensed in plastic pots with 1000 g capacity, and it was added the fungal

suspension ($1x10^7$ conidia per ml) for the pathogenic isolate in three replicates (18). After that, two seedlings of tomato plants four weeks aged were planted per pot of plastic. The plastic pots remained out for 6 weeks till emerged the symptoms, at which point the damage severity was graded using a scale of damage severity to study and test the pathogenicity of the studied isolate.

1. Rate of Wilting 0-23%
2. Rate of Wilting 24-50%.
3. Rate of Wilting is 51-75%
4 . Rate of Wilting is 76-100%.

The calculation of the injury's severity depends on the (17, 18) equation (1)

$$\text{Damage Severity\%}=\frac{\text{Total [Number of plants that infected} \times \text{The index of their pathology]}}{\text{[Total of plants that tested} \times \text{ the maximum value for pathologic evidence]}}\times 100 \quad (1)$$

2.3. Molecular identification

2.3.1.Extraction of genomic DNA

Genomic DNA was extracted from *F.incarnatum* isolated from infected tomato plants according to the protocol of ABIO Pure Extraction: This kit was used to quickly and easily isolate DNA from hard-to-lyse fungus. The isolate utilized in the DNA extraction was initially purified multiple times until any contamination (other fungus spores or bacteria) was removed by culturing the *Fusarium* isolate on the PDA medium for activation. The activation stage is crucial for DNA extraction to obtain pure cultures and a good product of genomic DNA (19).

2.3.2.Primers of gene *ITS*

The Macrogen Company provided these primers in lyophilized form. In a stock solution, lyophilized primers were dissolved in nuclease-free water to a final concentration of 100 pmol/µl. To make a workable primer solution containing 10 pmol/µl of these primers, 90µl of nuclease-free water was mixed with 10µ l of primer stock solution (stored at -20 C). The ITS sequences, which are found in all eukaryotes as a conserved region, were amplified using the universal primers (ITS-1 & ITS-4) (**Table 1**) (20, 21).

Table 1. The specific primer of gene *ITS.*

Primer Name	The sequence	Temp of annealing (°C)	Size of product
ITS 1	5`-TCCGTAGGTGAACCTGCGG-3`	55	570 bp
ITS 4	5`-TCCTCCGCTTATTGATATGC-3`	56	570 bp

2.3.3.PCR amplification

The PCR amplification was performed in a total volume of 25 µl using a thermal cycler, consisting of 3µl DNA, 12.5 µl of master mix (Promega, USA), 1µl of each primer, and 7.5µl of nucleease-free distilled water. The 35 cycles of amplification were as follows: initial denaturation, denaturation, annealing, and extension at 95°C for 5 min, 95°C for 30 min, 55°C for 30 sec, 72°C for 30 sec, and 72°C for 7 min, respectively. All products of PCR were separated utilizing 1.5% agarose gel electrophoresis, and the results were observed by ultraviolet light.

2.3.4.Phylogenetic analysis of *F. incarantum*

The ITS gene sequences obtained in the current research were compared to other fungal isolate sequences retrieved from the NCBI database by using nucleotide BLAST in Gen Bank .Evolutionary analysis were performed with MEGA6 (22,23).

3.Results

3.1. Morphological study of the Fungus

F.incarnatum isolate was obtained from samples of infected tomato plants. Pathogenic *Fusarium* isolates were purified and identified by using morphological and microscopic features. The morphological features of *F.incarnatum* isolate were observed on PDA media, such as a white cottony mycelium colony. Features of *F.incarnatum* observed under a microscope revealed that macroconidia have curved shapes, three to five septate, and basal cells with a foot shape and their dimension (17.59×3.25) µm. Microconidia are oval, without septa, and their dimension (7.98×3.93) µm in addition to the chlamydospores, as is seen in the culture (**Figure 1**).

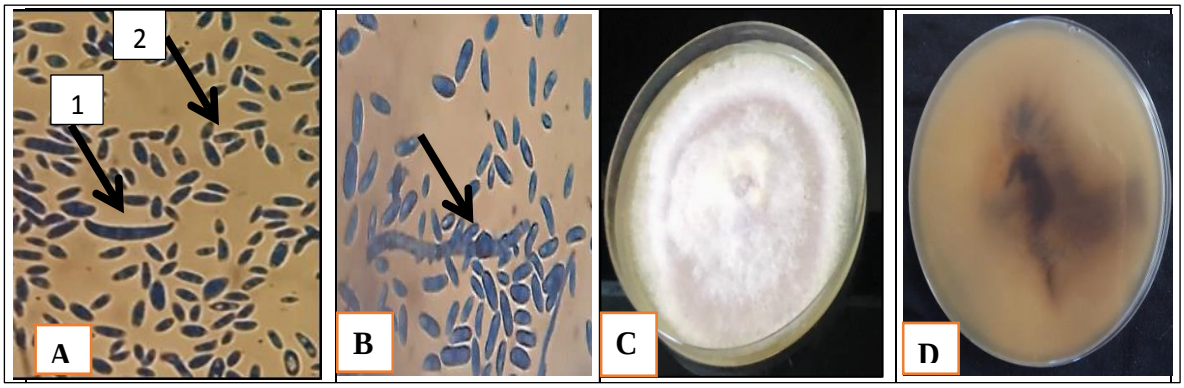

Figure 1. Morphological Identification of *F. incarnatum*, (A) (1) Macroconidia, (2) Microconidia under microscope, (B) Chlamydospore under microscope, (C) Morphology on PDA medium (Front view of the plate), (D) Morphology on PDA medium (back view of the plate).

3.2. Pathogenicity test

According to a pathogenicity study of the fungus *F. incarnatum*, the ability of the fungus to cause wilt disease in a healthy tomato plant was 90.33%. **Figure 2** illustrates healthy tomato plants that exhibited wilting symptoms, such as discolored vessels and yellowing leaves. The fungus was also isolated from the infected tomato plant to verify the characteristics of the fungal colony and the shape of the conidia of the fungus, and thus to ensure that the fungus that was isolated was the same one that was used for pathogenicity.

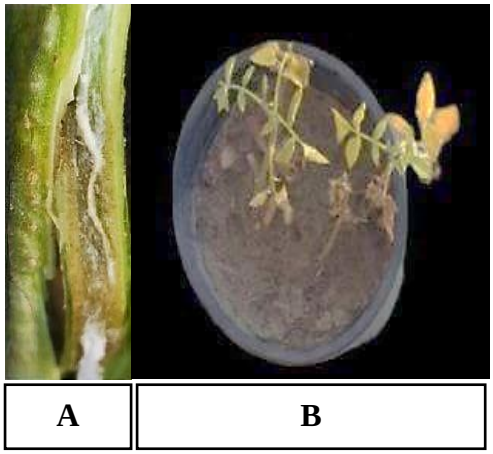

Figure 2. The pathogenicity of the fungus *F. incarnatum*, A. The symptoms of pathogenicity on stem, B. The symptoms of pathogenicity on whole plant.

3.3.Sequencing analysis of *ITS* region

The universal primers that were mentioned previously in the research were used to amplify the internal transcribed spacer region of the isolate to confirm the morphological identity; the amplified product likewise produced a single band in electrophoresis at 550 bp for the sample of *Fusarium* isolate following the PCR run, as shown in **Figure 3**. The isolated ITS rDNA

sequences have been presented to the database in the NCBI Gen Bank, and it obtained their accession number (OQ439282.1). The *Fusarium* isolate numbered (OQ439282.1) is similar at 100% to the *F.incarnatum* (OP006283.1).

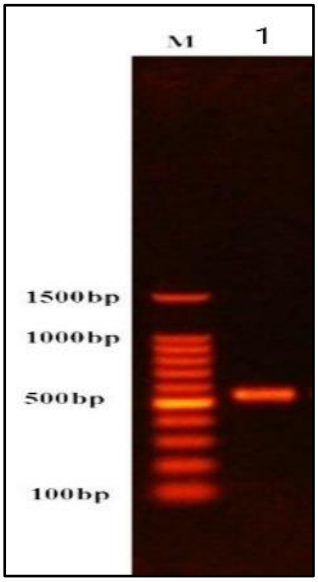

Figure 3. Result of the *ITS* region amplification of *F. incarantum* M: (100 – 1500) bp DNA ladder. Marker . Lanes .1 resemble to 570bp PCR Products.

3.4. Phylogenic analysis of *F. incarnatum*

The phylogenetic tree analysis is represented in Figure 4. The Iraqi isolate of this study **(**OQ439282.1**)** is shown in the same clade as the Turkey isolate (OP006283.1), India isolate (OP288161.1), and Chinese isolate (MW534570.1), and this clade shares 99.60% similarity with other clades, including Thai isolate, Taiwanese isolate, Nigerian isolate, South Korean isolate, Pakistani isolate, Japanese isolate, Egypt isolate, American isolate, and Tunisian isolate (MT796350.1, MZ749694.1, MN882828.1, KY508359.1, MT080367.1, AB975304.1, OW983261.1., GQ505680.1, MT312734.1, respectively).

Table 2. Shows the global sources of *F.incarnatum* isolates that were used in comparison with the Iraqi isolate in this research, these sources are from the database of NCBI

Source*: F. incarnatum*			
Accession	**Country**	**Isolation source**	**Compatibility**
ID: OP006283.1	Turkey	Pepper	100%
ID: OP288161.1	India	Cinnamon	100%
ID: MW534570.1	China	-----	100%
ID: MT796350.1	Thailand	------	99%
ID: MZ749694.1	Taiwan	Musk melon	99%
ID: MN882828.1	Nigeria	Dried food	99%
ID: KY508359.1	South Korea	sweet potato	99%
ID: MT080367.1	Pakistan	Pumpkin	99%
ID: AB975304.1	Japan	------	99%
ID: OW983261.1	Egypt	Lycopersicon esculentum	99%
ID: GQ505680.1	USA	--------	99%
ID: MT312734.1	Tunisia	Soil from industrial area	99%

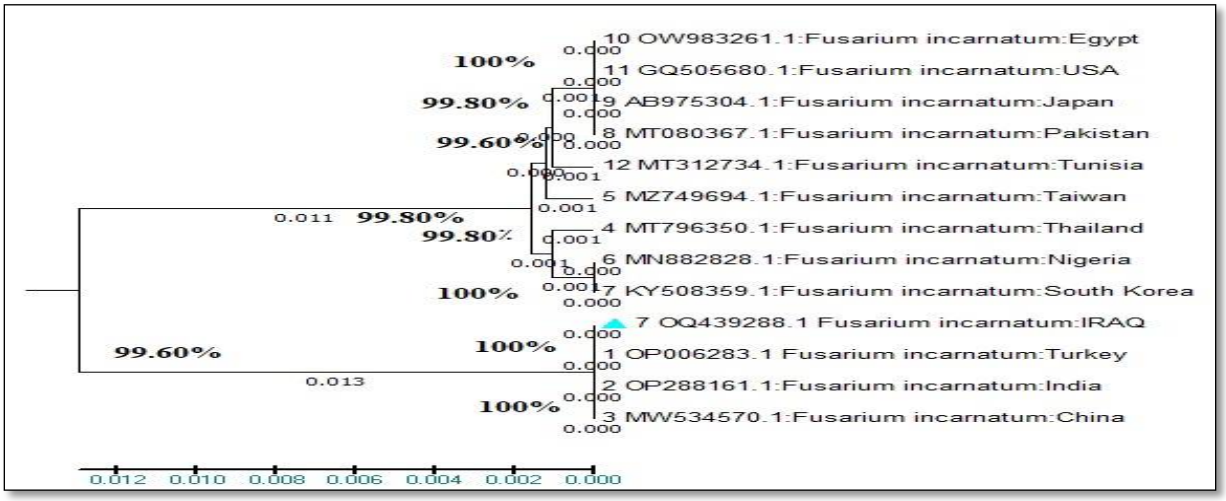

Figure 4. The phylogenetic tree was generated using *ITS* region nucleotide sequence information for *F.incaranatum* (Iraqi isolates in this study compared with other global isolates). The evolutionary history was inferred using the MEGA6 method.

4.Discussion

For the first time, *F. incarnatum* has been identified as the causal agent of tomato wilt in Iraq. This has significant implications for tomato production in the region. Tomatoes are one of the most important vegetable crops in Iraq, and the emergence of *F.incarnatum* poses a new challenge for farmers. Early and accurate identification of such pathogens is critical for the development of targeted disease management strategies. The integration of molecular diagnostics with traditional morphological methods can improve monitoring programs and reduce the risk of misdiagnosis, which often leads to ineffective control practices. These morphological characteristics were identical to those of *F.incarnatum* that were mentioned by (3, 4). Numerous plants across the world have been documented to be infected by *F. incarnatum* (7, 8). However, no official reports of *F. incarnatum* causing wilt on tomato plants in Iraq have been made (24, 25, 26).

Research and studies have shown that numerous pathogenic fungi, whether pathogenic to plants or existing in the soil, can be revealed and identified by using molecular biology techniques, particularly PCR (15). The analysis of sequence is the best choice for phylogenetic research in the *Fusarium* species Since the ITS rDNA is being studied more frequently, it has been demonstrated to provide the optimum resolve at the sub-species level. (27-29). According to previous investigations, molecular analysis is typically chosen for the characterization and identification of *Fusarium* species since it makes it simple to identify the differences between them. For an accurate diagnosis of *Fusarium*, morphological examinations and molecular characteristics must be combined (30). One of the main pathogenic genera that affect tomatoes is *Fusarium.* The growth of toxigenic fungal species, the expression of biosynthetic regulatory genes, and the generation of mycotoxin are all impacted by the interaction of environmental stress factors like temperature and water activity (31). In this study, *F. incarantum* was identified as the causal agent of wilt disease in the tomato plant in Iraq, which is crucial in reducing the risk of the pathogen spreading to other regions. Additionally, this will provide us with pathogen-related biological management strategies and enable us to use phytosanitary techniques to stop the disease's spread.

5. Conclusion

Among the most significant pathogens for plants in the world are *Fusarium* species. While many *Fusarium* species are widely known, other pathogenic species are currently undiscovered or characterized. Accurate identification of *Fusarium* species is achieved by combining molecular techniques and morphological methods. The causative agent of the tomato plant wilting disease was determined to be *F. incarnatum* based on morphological characters and *ITS* sequencing data. In this study, *F. incarantum* was identified as the causal agent of wilt disease in the tomato plant for the first time in Iraq, which is crucial in reducing the risk of the pathogen spreading to other regions. Additionally, this will provide us with pathogen-related biological management strategies and enable us to use phytosanitary techniques to stop the disease's spread.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Funding

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Ethical Clearance

The research was not subject to research ethics as it included conducting a survey of the species of the *Fusarium* genus infect tomato plant in Iraq.

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