Research Article

First Report on Morpho-Molecular Identification of Pathogenic Fungal Isolates Associated with Rosemary (*Rosmarinus officinalis* L.) Plants in Al-Qadisiyah, Iraq

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Abstract | *Rosemary (Rosmarinus officinalis L.) i*s one of the important ornamental, aromatic, and medicinal plants belonging to the family *Lamiaceae. This plant* is infected by several plant pathogens, causing considerable economic losses to its growth and production. *Among these, Fusarium wilt* is a major and destructive disease hampering rosemary cultivation in many agroclimatic settings in Iraq. This study aimed to identify the fungal pathogens associated with the rosemary seedlings showing wilt symptoms. To this end, infected rosemary plants were collected randomly from different local nurseries of Al-Qadisiyah Governorate of Iraq. Fungal pathogens from the roots of these plants were isolated, propagated, and identified based on the morphological characteristics of colonies (*i.e.*, colony color, mycelial growth, and colony diameter) and confirmed by molecular sequencing. The initial morphological characterization showed the presence of two pathogenic fungal species, *i.e., Fusarium solani* and *F. chlamydosporum*. Furthermore, molecular diagnosis based on sequencing of the ITS-DNA gene region using ITS1F and ITS4 R primers corroborated the morphological diagnosis. Sequence data analyses exhibited 99 and 100% alignment of Iraqi isolates of *F. solani* and *F. chlamydosporum, along* with the Chinese isolates (MN960013.1 and KX783373.1), respectively. DNA sequences of Iraqi isolates of *F. solani* and *F. chlamydosporum* are deposited in the NCBI Gene-Bank with accession codes OQ299566.1 and OQ301746.1, respectively. To our knowledge, this study encompasses the first morphological and molecular diagnosis of the pathogenic fungal species responsible for *Fusarium* wilt in rosemary plants in Iraq, posing a serious threat to rosemary cultivation in nurseries.

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Keywords | Rosemary seedlings, *Fusarium* wilt, *Fusarium solani*, *Fusarium chlamydosporum*, Morphological identification, Molecular characterization

Introduction

Rosemary (*Rosmarinus officinalis* L.) is a significant perennial evergreen shrub that belongs to the Lamiaceae family. It is an herbaceous plant indigenous to Mediterranean areas, widely cultivated throughout the world for its aromatic, medicinal, and ornamental features (Pintore *et al.*, 2002; Hammer and Junghanns, 2020). Essential oil of rosemary is frequently used as a spice and flavoring agent and is famous for its antimicrobial, anti-inflammatory, and antioxidant activities in traditional medicine (Lo *et al.*, 2002; Becer *et al.*, 2023). Moreover, rosemary essential oil contains many bioactive compounds such as rosmarinic acid and Rosmanol that have many therapeutic uses against different human diseases, including cancer, atherosclerosis, and inflammatory diseases (Rafie *et al.*, 2017; Becer *et al.*, 2023, Bakrim *et al.*, 2025).

Several biotic factors, such as plant pathogens, can affect the rosemary growth and production. The soilborne fungal diseases, such as *Fusarium* wilt, impose substantial damage to rosemary seedlings. Mycelium, spores, or polysaccharides of *Fusarium* fungus may obstruct xylem tissue, causing wilt, a condition that shows up as a sharp decrease in water flow to plant tissues (Saremi *et al.*, 2011). Discoloration of the vascular bundle originating from roots to aerial parts is the main symptom of *Fusarium* wilt that ultimately causes crown and root rot, defoliation, and death of the plant (Mekonnen, and Manahile, 2017; Mekonnen *et al.*, 2019).

Fusariosis, plant diseases caused by different phytopathogenic strains of *Fusarium* species, cause substantial economic losses to plants worth billions of USD (Ekwomadu and Mwanza, 2023). These fungal pathogens are ubiquitously found throughout the world in soil, water, plants, and insects (Sharma and Marques, 2018). They may spread from the affected fields through irrigation and crop residues (Mekonnen *et al.*, 2019). Some of the phytopathogenic *Fusarium* species cause infection in rosemary plants, including *F. solani*, *F. oxysporum*, *F. reticulatum,* and *F. sambucinum* (Ashrafi *et al.*, 2010; McGovern, 2023).

Pathogenic *Fusarium* species are usually identified based on the morphological characters of their mycelia and colony shape and growth pattern (Harish *et al.*, 2023). The size and shape of macro- and micro-conidia, in addition to the presence or absence of chlamydospores, are the main morphological traits used to distinguish *Fusarium* species (Leslie and Summerell, 2006; Chandana *et al.*, 2024). Moreover, internal transcribed spacer (ITS) segments of the ribosomal DNA sequences have recently been used as a molecular marker for systematics as well as phylogenetic analysis of closely related fungi (Schoch *et al.*, 2012). ITS region sequencing has been effectively employed for the fungal species' characterization (Nilsson *et al.*, 2009).

Based on the above-mentioned information, this study aimed to identify the Fusariosis-causing pathogenic fungi associated with rosemary seedlings based on their morpho-molecular traits. Although limited research has been conducted in Iraq to identify the soilborne *Rhizoctonia solani* associated with rosemary (Al-Taae and AL-Taae, 2020), there has been a lack of research efforts in investigating root rot caused by *Fusarium* species on rosemary plants in Iraq. Therefore, the purpose of this research work was to isolate and diagnose the cause of rosemary wilt in Iraq caused by *Fusarium* species using both morphological and molecular methods.

Figure 1: *Fusariosis symptoms on rosemary plant showing symptoms of wilting and yellowing, and crown and root rot.*

Materials and Methods

Plant samples

Rosemary seedlings exhibiting different symptoms of *Fusarium* wilt, including crown and root rot, and darkened vascular tissues of leaves (Figure 1), were sampled and collected randomly from different plant nurseries located in Al-Diwaniyah city of Al-Qadisiyah province of Iraq. Plant specimens were assembled and sent to the laboratory for pathogens' isolation under cool conditions. The infected roots were chopped into tiny pieces, each measuring 0.5 to 1.0 cm in length, and were surface-sterilized for 2 min using 0.1% sodium hypochlorite and then

washed with sterilized distilled water. Disinfected filter papers were used to dry the treated samples, which were then incubated on potato dextrose agar (PDA) medium in sterilized glass Petri-plates (60 cm diameter) at 25°C for a period of 7 days. To create a genetically pure culture, the hyphal extremity of the mycelia was re-cultured on PDA. The identification of the cultured pathogens was done by studying morphological characteristics of isolates under a compound microscope (up to 100×) (Leslie and Summerell, 2006).

Molecular detection and identification

Morphological and genetic traits were used to identify the *F. solani*, *F. chlamydosporum*, and *A. terreus* that were isolated from the infected rosemary samples. Using the collected mycelia, genomic DNA extraction and PCR amplification were carried out. ZR-Fungal/Bacterial/Yeast DNA MiniPrep Kit (ZYMO, USA) and FavorPrep Tissue Genomic DNA Extraction Mini Kits (Favorgen Biotech Corp, Ping-Tung, Taiwan) were used to extract the fungal DNA. An ITS gene fragment was amplified using ITS1/ITS4 forward "TCC GTA GGT GAA CCT TGC GG 3" and the reverse primer "TCC TCC GCT TAT TGA TAT GC 3" (Zarrin *et al.*, 2016; Rashid, 2025).

In brief, a PCR reaction volume of 25 µl contained 5 µl Taq PCR PreMix (Intron, Korea), 1.5 µl target DNA, 1 µl of primer (10 pmol), and distilled water. PCR amplification was performed on a thermal cycler (Gene Amp, PCR systems 9700; Advanced Biosystem) using the following thermal protocol. Three min of denaturation at 94°C, thirty-five cycles of dehydration at 94°C for forty-five sec, one min of annealing at 52°C, one min of extension at 72°C, and a final seven-min incubation step at 72°C. The final PCR products were visualized under a UV lamp on a 1.5% agarose gel with Red Safe Stain (20,000× Intron).

Sequencing of the PCR products of each fungal isolate was carried out by Macrogen, Inc. (Seoul, Republic of Korea) using an ABI 3730 XL Automatic Sequencer. Using previously deposited sequences from the National Center for Biological Information (NCBI), BioEdit Sequencing Alignment Editor (Version 7.1; DNASTAR, Madison, WI, USA) aligned pertinent isolated sequences. As indicated in Table 1, the isolated sequences of *F. solani*, *F. chlamydosporum*, and *A. terreus* used in this study were uploaded to GenBank with accession codes MN960013.1, KX783373.1, and MH918670.1, respectively.

Results and Discussion

Cultivation of rosemary, one of the popular medicinal and ornamental plants worldwide, is hampered by many soilborne diseases. Among these, Fusariosis is the most destructive disease often caused by *Phytophthora*, *Fusarium*, and *Rhizoctonia* species (Jambhulkar *et al.*, 2015; Ekwomadu and Mwanza, 2023). Particularly, *Fusarium* species impose substantial damage to different plants, including rosemary (Ashrafi and Saremi, 2012; Mekonnen and Manahile, 2017).

In this study, pathogenic fungal isolates of the wilted rosemary plants were cultured on PDA and were identified on the basis of their morpho-cultural traits as shown in Figure 2, 3, and 4. Results of morphological

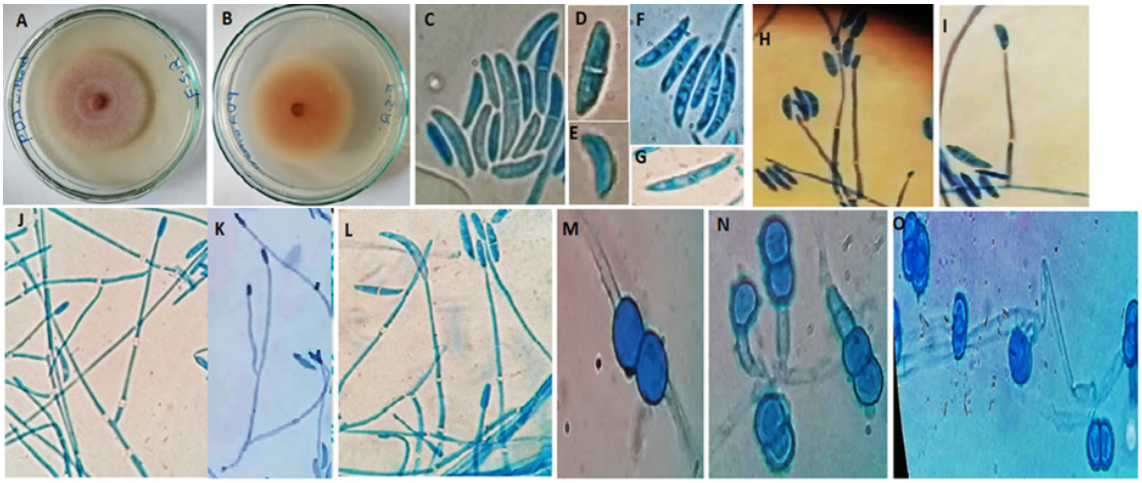

Figure 2: *Morpho-cultural traits of Fusarium solani causing wilting and rot of rosemary (Rosmarinus officinalis) plants.*

diagnosis showed two species of *Fusarium* that caused fusariosis in rosemary plants. The first species was *Fusarium solani*, which distinguished itself with its appearance in PDA at 25°C, and showed pink upper-surface colonies and yellowish-brown pigmentation on the lower surface (Figure 2 A–B) with a radius of 50.5 mm after 7 days of growth. Microconidia observed were of oval or ellipsoid shape, consisting of a single cell. Additionally, there were sub-cylindric or slightly curved microconidia, consisting of two cells (Figure 2 C–E). The aerial conidiophores exhibited a lengthy and unbranched morphology that was somewhat narrow towards the apex. These structures were monophyletic and produced a plentiful supply of 0–1 conidia, which were clustered in a false head. (Figure 2 H–J). Macroconidia were slender to relatively straight, 3–5-septa (Figure 2 F–G). Lateral-branched aerial conidiophores producing 1–4 septate conidia (Figure 2 K–L). Intercalary chlamydospores present in hyphae were typically characterized by smooth walls and a globose shape, and were found in pairs (Figure 2 M). Terminal chlamydospores from conidiophores were smooth-walled and globes singly, in pairs, or in clusters (Figure 2 N–O). Similar features of mycelial growth and colony patterning of *F. solani* on PDA medium are reported by Zidan *et al.* (2022) and Valadez-Moctezuma *et al.* (2025).

The second species was *F. chlamydosporum*, characterised by a white cottony upper surface of colony and creamy-yellow pigmentation of the lower surface on PDA after 7 days (Figure 3 A–B) with a colony radius of 70 mm. Spindle-shaped microconidia with 0–1 septation (Figure 3 C) and typical sickle-shaped macroconidia with 3–5 septa (Figure 3 D–E) were observed. Microconidiophores were observed to manifest as both unbranched and branched monophialides, as well as polyphialides conidogenes, as depicted in Figure 3 (F–H). In addition, globose-shaped clamydospores were observed and were formed in either singular or short chain formations, as shown in Figure 3 (I–K). Current results confirmed the findings of Gupta and Misra (2012) and Lazreg *et al.* (2013), and Ibrahim *et al.* (2016).

In addition to *Fusarium* species, *A. terreus*, a significant saprophytic filamentous fungus found in the soils, was also characterised by cinnamon colored colony (Figure 4). Animals, plants, and humans can all be harmed by the filamentous fungus *A. terreus* (Nji *et al.*, 2023). More recently, *A. terreus* has also been found causing foliar blight of potato and may infect other crops (Louis *et al.*, 2013). (Louis *et al.*, 2013; Al-Baldawy *et al.*, 2020; Pennerman *et al.*, 2020). Morphological characters of both *Fusarium* species were confirmed by ITS gene-based molecular markers (Zarrin *et al.*, 2016). The size of the PCR products was about 600 bps. Molecular phylogenetic methods have been extensively employed to facilitate the accurate species identification of the *Fusarium* genus (Chehri *et al.* 2015; Stoeva *et al.*, 2023). Using the NCBI blast web, the sequence alignment of the *F. solani* isolate revealed a 99% identity with the *F. solani* Chinese isolate (accession No. MN960013.1),

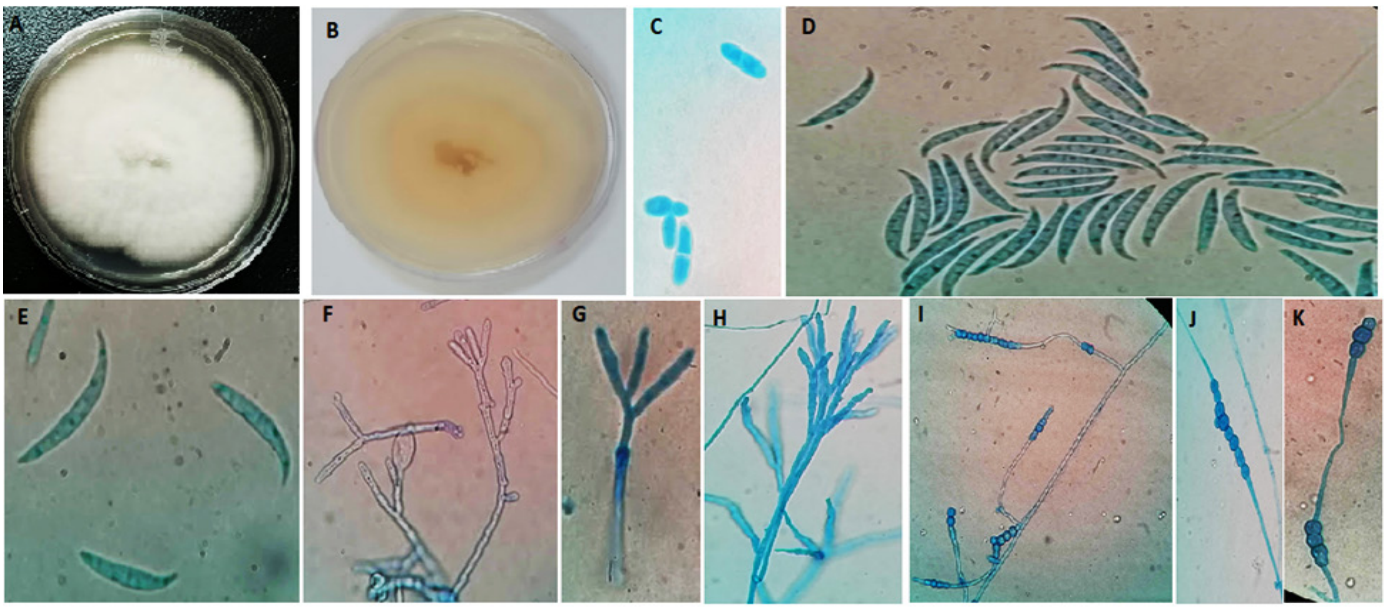

Figure 3: *Morpho-cultural traits of Fusarium chlamydosporum causing wilting and rot of rosemary (Rosmarinus officinalis) plants.*

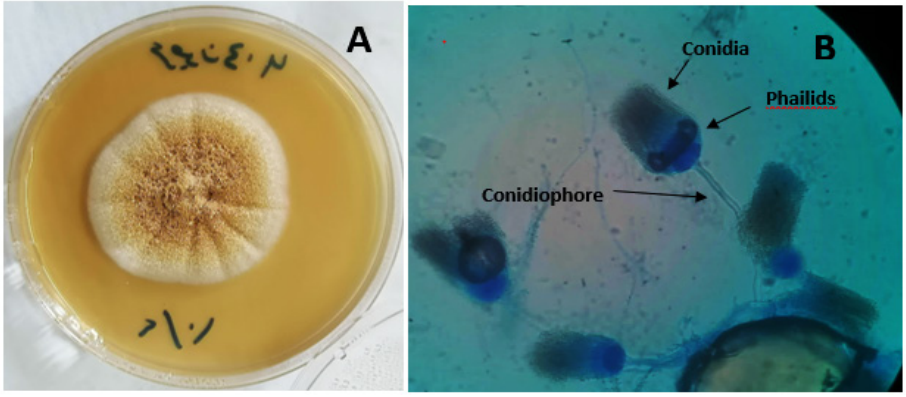

Figure 4: *Morpho-cultural traits of Aspergillus terreus causing wilting and rot of rosemary (Rosmarinus officinalis) plants*

Fusarium solani isolate 31 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

Sequence ID: MN960013.1 Length: 539 Number of Matches: 1

Range 1: 57 to 522 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
839 bits(454)	0.0	460/466(99%)	0/466(0%)	Plus/Plus

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Query  1    AACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGACCCCCTAACTCTGTTTTTAT  60
Sbjct  57   AACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGACCCCCTAACTCTGTTTTTAT  116
Query  61   AATGTTTTTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGC  120
Sbjct  117  AATGTTTTTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGC  176
Query  121  TCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT  180
Sbjct  177  TCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT  236
Query  181  GAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTT  240
Sbjct  237  GAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTT  296
Query  241  CGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAGCGCCCC  300
Sbjct  297  CGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAGCGCCCC  356
Query  301  CTGCGGGCACACGCCGTCCCCCAAAKMCAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTA  360
Sbjct  357  CTGCGGGCACACGCCGTCCCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTA  416
Query  361  GTAGCTRACACCTCGCAACTGGAGAGCGGCGCGGCCACGCCGKAAAACRCCCAACTTCTG  420
Sbjct  417  GTAGCTAACACCTCGCAACTGGAGAGCGGCGCGGCCACGCCGTAAAACACCCAACTTCTG  476
Query  421  AATGTTGACCTCGAATCAGGKAGGAATACCCGCTGAACTTAAGCAT  466
Sbjct  477  AATGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTTAAGCAT  522
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Figure 5: *Alignment of partial ITS gene sequences of the Iraqi isolate of Fusarium solani (accession no. OQ299566.1) along with the Chinese isolate (accession no. MN960013.1) using NCBI-BLAST. The query and the subject sequences represent the Iraqi and Chinese isolates, respectively.*

with six variation sites, *i.e.,* K (G,T) > T and M (A,C) > A in 382 bp, 384 bp, but in 423 bp, 459 bp, and 465 bp. The study revealed evidence of both transition mutations, which involve the substitution of a two-ring purine for a one-ring pyrimidine or vice versa. Specifically, the locations 423, 459, and 465 bps exhibited the occurrence of transition R (A, G)>A, R (A, G)>A, and R (A, G)>A, respectively. Additionally, transversions were also observed in locations 382, 383, and 497 bps (Table 1 and Figure 5).

A phylogenetic tree of the Iraqi isolate of *F. solani* was drawn to illustrate the relationship between the species that were deposited in the GenBank database based on the ITS gene region by using the Mega 6 program, which illustrated the relationship of *F. solani* with five sequences of global isolates in GenBank, which were divided into two main nodes (A and B). A node included three isolates 100% similarity, but two isolates, Iraqi isolate (OQ299566.1) and Spanish isolate (LN809053.1), with 94% compatibility. B node contained three isolates, *i.e.,* OM876905.1, JQ910159.1, and OQ345527.1 (Figure 6).

Similarly, a comparison between the sequence of *F. chlamydosporum* Iraqi isolate (OQ301746.1) and *F. chlamydosporum* Chinese isolate (KX783373.1) showed 100% identity, with no evidence of substitution in their respective positions (Table 1).

The phylogenetic tree of *F. chlamydosporum* Iraqi isolate (OQ301746.1) illustrates its relationship with five sequences of global isolates deposited to the GenBank database (Figure 7). Relationships

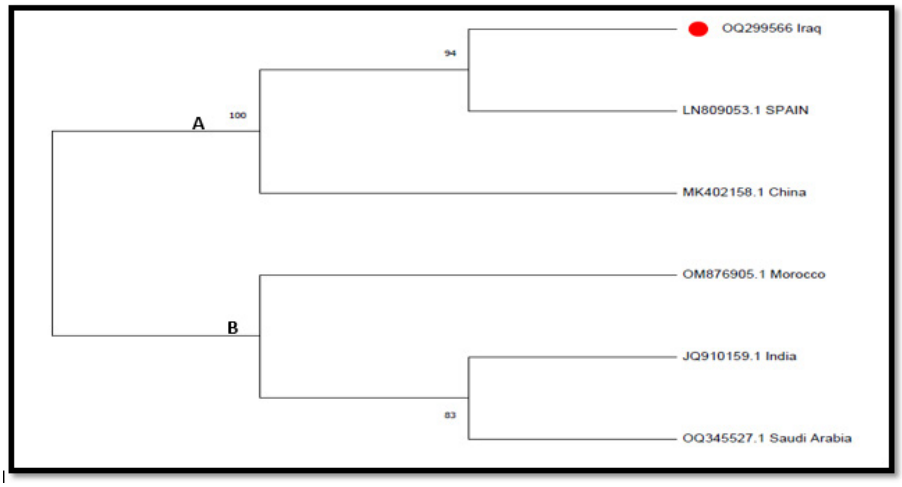

Figure 6: *Dendrogram produced by the analysis of ITS sequencing data depicting the evolutionary relationship and genetic similarity of the pathogenic fungal Fusarium solani isolates.*

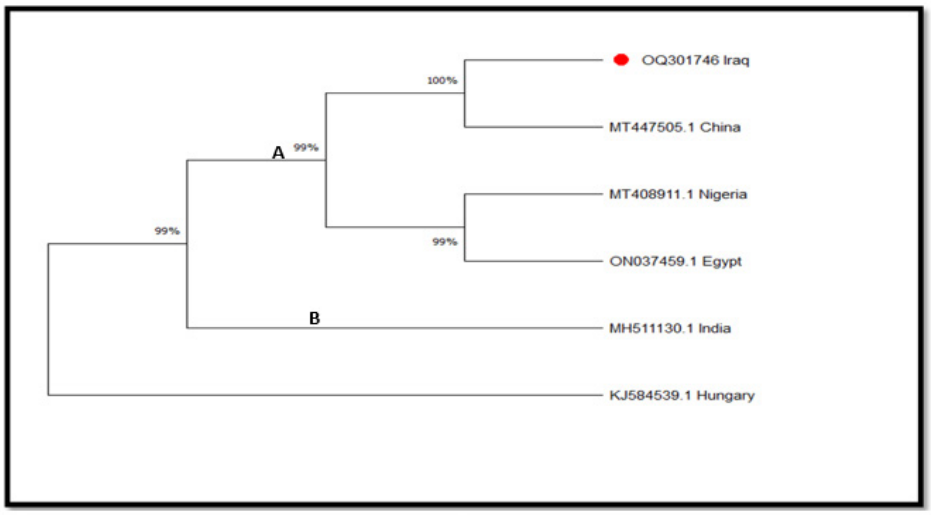

Figure 7: *Dendrogram produced by the analysis of ITS sequencing data depicting the evolutionary relationship and genetic similarity of the pathogenic fungal Fusarium chlamydosporum isolates.*

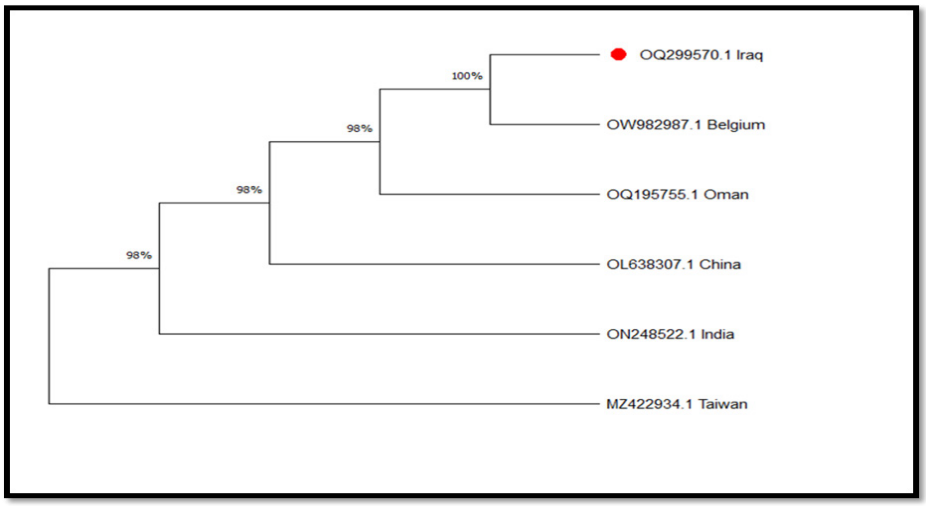

Figure 8: *Dendrogram produced by the analysis of ITS sequencing data depicting the evolutionary relationship and genetic similarity of the pathogenic fungal Aspergillus terreus isolates.*

were divided into two branches; the first branch was divided into two main nodes (A and B). Node A included two internal nodes, first internal node included two isolates, *i.e.*, Iraqi isolate (OQ301746.1)

Table 1: *Kind of ITS region polymorphism for Aspergillus terreus, Fusarium species, and Iraqi isolates. Transition & transversion mutations are compared to sequences in the GenBank database.*

Iraqi isolates	Identity	GenBank isolates	Sequence ID with compare	Nucleotide	Location	Type of situation
***F.solani* OQ299566.1**	99%	*F. solani* isolate 31	MN960013.1	(G,T)K >T	382bp	Transversion
				M (A,C) > A	383bp	Transversion
				R(A,G) >A	423bp	Transition/ Transversion
				K(G,T) >T	459bp	Transition/ Transversion
				R(A,G) >A	465bp	Transition/ Transversion
				K(G,T) >T	497bp	Transversion
***F.chlamydosporum* OQ301746.1**	100%	*F.chlamydosporum* isolate ZB11263540	KX783373.1	-	-	-
***A.terreus* OQ299570.1**	100%	*A. terreus* strain CA-1	MH918670.1	-	-	-

and Chinese isolate (MT447505.1), with 100% compatibility. Second internal nodes were included Nigerian isolate (MT408911.1) and Egyptian isolate (ON037459.1) with 99% compatibility. Finally, the second internal node (B) were Indian isolate (MH511130.1) compatible with four previous isolates with 99% compatibility. The second branch included a Hungarian isolate (KJ584539.1) showing 99% compatibility with five isolates (Figure 7). Moreover, the identity ratio of the sequences of Iraqi isolate of *A. terreus* (OQ299570.1) was 100% with *A. terreus* Indian isolate (MH918670.1) (Table 1). Dendrogram produced by the analysis of ITS sequencing data depicted that the Iraqi isolate of *A. terreus* (OQ299570.1) had a compatibility of 100% with Belgium isolate (OW982987.1) (Figure 8).

Conclusions and Recommendations

Based on overall study results, it is concluded that Fusariosis or *Fusarium* wilt affecting the rosemary cultivation and production in the nurseries of the sampled area of Iraq is primarily caused by two species of pathogenic fungal isolates, *i.e., F. solani* and *F. chlamydosporum*. Moreover, the findings corroborate the practical significance and implications of morpho-molecular diagnosis of plant pathogens. These results would aid in understanding the key factors that contribute to the spread of *Fusarium* wilt of rosemary plants, and in figuring out the most effective ways to control it in light of the present study's results.

Acknowledgments

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Novelty Statement

This study proposes *Fusarium wilt* is a major and destructive disease hampering rosemary growing in different areas in Iraq.

Author's Contribution

All authors contributed to data collection and edited the draft and final versions of the manuscript.

Generative AI and AI-assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest
The authors have declared no conflict of interest.

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