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FIRST REPORT OF *EXSEROHILUM ROSTRATUM* AS A POTENTIAL PATHOGEN OF THE FABA BEAN LEAF SPOT DISEASE IN IRAQ

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SUMMARY

Faba bean (*Vicia faba* L.) is one of the foremost vegetable crops in Iraq and worldwide. Faba bean has also become a traditional food in different parts of the world, with cultivations mainly for their protein-rich pods. Faba beans sustain several fungal pathogen infections, which lead to considerable yield losses. Among these, the leaf spot disease is more prominent and considerably impacts the quality and quantity of faba bean production. In Iraq, the leaf spot disease has emerged as a significant problem in bean fields caused by several pathogens. In the presented work, sizable efforts focused on isolating and identifying fungal pathogens of the leaf spot disease in faba beans at the Basrah Governorate, Iraq. The study might also be the first report on the fungal species *Exserohilum rostratum* as a true pathogen of faba bean leaf spot disease in Iraq. The morphological and molecular diagnoses identified the pathogen by applying the internal transcribed spacer (ITS) gene**.** Searching the sequenced PCR products used the NCBI-BLAST website. The results proved a 99% similarity to the known fungus *E. rostratum,* with an eventual submission to NCBI under the gene accession number LC769969. The pathogenicity experiment materialized following Koch's hypotheses to confirm the causative agent. The presented findings revealed the potential pathogenicity of this microbe on the aerial parts of the faba bean (*V. faba* L.) for the first time in Iraq.

Keywords: Faba bean (*V. faba* L.), *Exserohilum rostratum*, leaf spot disease, morphological and molecular identification, pathogenicity

Key findings: The appropriate study identified the fungal species *Exserohilum rostratum* as a potential threat in cultivating faba bean (*V. faba* L.) in Iraq. This research represents the first report of its pathogenic effect on aerial parts (stems, leaves, and pods) of the faba bean plants in Iraq.

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INTRODUCTION

The faba bean (*V. faba* L.) is one of the leguminous crops belonging to the family Fabaceae. It is a crop rich in protein and other essential chemical compounds in the green and dry pods and leaves. Among these chemicals, the phenolic compounds, flavonoids, biologically active peptides, resistant starch, dietary fiber, beta-aminobutyric acid (GABA), and l-3,4-dihydroxyphenylalanine (L-DOPA) proved mainly responsible for various biological activities, such as antidiabetic, anti-inflammatory, hypotensive, anti-inflammatory, antiviral, antibacterial, antioxidant, antimalarial, anticancer, and cholesterol-lowering (Abed *et al*., 2022; Jaafar *et al*., 2022**;** Kumar *et al*., 2022).

Despite being a highly versatile and nutritious crop, some regions of the world remain unaware of faba beans' potential benefits, as well as their remarkable resilience to global warming and climate change and ability to adapt well to various climatic conditions and diverse soil environments (Singh *et al*., 2013). Plants widely incur infections with several fungal pathogens, which lead to significant yield losses for quality and quantity levels at different growth stages, with more than 19,000 fungal species causing these diseases in crop plants worldwide (Jain *et al*., 2019).

The fungi can remain dormant but always viable on living and dead plant tissues until favorable conditions allow them to multiply. Some fungi also can develop and thrive within the tissues of their host plants. Fungal spores can easily spread by various means, i.e., wind, water, soil, insects, and other invertebrates. Consequently, these pathogens can potentially infect entire crops and cover large distances, contributing to widespread infestations (Lazarovits *et al*., 2014). Several fungi can considerably infect the vegetative system of plants, causing significant diseases, like *Alternaria* spot, powdery mildew, downy mildew, *Fusarium* wilt, rusts, and various others (Jain *et al*., 2019; Garibaldi *et al*., 2023)**.**

Faba bean plants sustain infections at different growth stages, with varied pathogens like bacteria, fungi, viruses, and numerous others, leading to significant production losses (Plūduma-Pauniņa *et al*., 2019). The diseases that infect vegetative parts are often severe in faba bean, such as chocolate spot (*Botrytis fabae*), *Ascochyta* blight (*Didymella fabae*), rust (*Uromyces viciae-fabae*), *Cercospora* leaf spot (*Cercospora zonata*), *Stemphylium* leaf blight (*S. botryosum*, *S. eturmiunum,* and *S. vesicarium*) (Stoddard *et al*., 2010; Bankina *et al*., 2017; Vaghefi *et al*., 2020). However, a report stated the *Exserohilum rostratum* species is a potential threat to numerous valuable plant species which belonging to 28 genera within 11 families (Vaghefi *et al*., 2020).

The numerous affected crop plants that are economically valuable include corn, rice, sugarcane, sweet sorghum, tomato, and wheat. The fungus' ability to impact a wide range of hosts highlights its adaptability and significance in affecting diverse types of crop plants (Cardona and González, 2007). According to the best knowledge of the authors, no previous study has shown the pathogenicity of *Exserohilum rostratum* on faba bean leaves as a leaf spot disease in Iraq; therefore, the presented research will be the first report on *E. rostratum* to cause the leaf spot disease in faba beans. The study aimed to isolate and characterize the *E. rostratum* as a new pathogen for leaf spot disease in faba beans.

MATERIALS AND METHODS

Samples collection and fungal isolation

Faba bean (*Vicia faba* L.) samples of symptomatic plant material, including leaves and stems, commenced collection during the fourth quarter of 2022 and the first quarter of 2023 at the Basrah Province from different regions, including Shat-Al-Arab and Abu-Alkhasseb in Iraq. In the laboratory, the procured samples' washing used running water and sterile distilled water, then cutting them into small pieces measuring 0.5–1 cm in length. All fungi and bacteria removal already found on the faba bean plant parts and surface

employed a 5% NaOCl solution for three minutes. The samples received rinsing twice with sterile distilled water to remove any residual sterilizing solution. Subsequently, the plant parts' drying used sterilized filter paper (Abass, 2016).

For cultivation purposes, preparing 9-cm Petri dishes containing the standard PDA (potato dextrose agar) supplied by Oxoid Ltd. culture medium ensued. Each plate, inoculated with four pieces of infected plant tissues and three plates, contained each portion of the faba bean plants. Each Petri dish proceeded in an incubator at 25 °C ± 2 °C for five to seven days to allow fungal growth on the culture medium. Purifying the developing fungal colonies helped obtain pure fungal isolates. A sterilized needle, treated with alcohol and flame, extracted a piece of the fungus growing around the plant parts. Transferring the isolated portions to Petri dishes containing PDA culture medium followed, with the plates incubated under the same conditions (as before) to ensure the growth of uncontaminated fungal isolates (Yin *et al.*, 2017; Razaq and Abass, 2021).

Morphological identification of isolated fungi

Documenting the morphological characteristics of the isolated fungi comprised aseptic 5-mm diameter discs extracted from the pure colonies after seven days of growth at 25 °C ± 2 °C. These discs, placed at the center of Petri dishes containing PDA medium, reached incubation in an incubator. The fungi's examination had their morphological features recorded using naked-eye observations and a compound light microscope. It involved noting the form, size, margin, transparency, and colony color of the isolated fungi. Furthermore, the developing fungi's scrutiny under a light microscope determined the color of the fungal mycelium and spore-bearing structures (conidia). The shape, color, size, and other microscopic characteristics relevant to their classification proceeded to document. Detailed recording of the phenotypic and morphological characteristics of the isolated and identified fungi was meticulous. Preserving fungal cultures consisted of discs taken from the edges of the colonies using a sterile ring conveyor and then used to inoculate solid-slanted culture media. These preserved isolates as slant cultures attained storage at 4 °C in a refrigerator until required for subsequent studies (Ellis, 1971, 1976; Tang *et al*., 2023).

Molecular identification of the pathogens

The molecular identification followed these steps: utilizing the pure mycelium isolates, conducting molecular diagnostic experiments using the internal transcribed spacer (ITS) region of the fungal isolates' DNA, and employing the polymerase chain reaction (PCR). The experiments began using the sterile ring conveyor filler to obtain samples from each test tube containing the pure fungal isolates. These samples sustained inoculation onto Petri dishes containing solid culture medium (PDA) and had three replicates for the fungal species. Subsequently, the Petri dishes in an incubator had a temperature of 25 °C ± 2 °C for seven days, allowing the fungal colonies to grow and provide further extraction of the genetic material, specifically DNA.

Following the growth period, genomic DNA extraction from the fungal colonies ensured obtaining the DNA required for the molecular diagnosis. The PCR conditions necessary amplify the extracted DNA from the fungal isolates (Kim *et al*., 2001, 2017). The PCR reactions resulted in a final concentration of 25 µL. The efficiency of the PCR reaction runs the amplicon DNA fragment on 1.5% agarose gel electrophoresis. Details of the primer sequences employed in the PCR are available in Table 1, with the PCR conditions outlined in Table 2. The PCR products obtained subsequently underwent sequencing according to the instructions of Macrogen Company, South Korea (http://dna.macrogen.com) for handling the preparations and samples. The ITS gene sequence, submitted to GenBank (https://www.ncbi.nlm.nih.gov/), attained the accession number LC769969 and was analyzed using the BLAST search tool. Similar sequences, downloaded from a database at GenBank and aligned, employed Clustal

Table 1. The ITS primer sequence applied in the PCR amplification.

Primer	Primer sequence	Length of PCR product	Reference
ITS-1	5'- TCCGTAGGTGAACCTGCGG - 3'	550-600 (bp)	(Yin *et al*., 2017)
ITS-4	5'- TCCTCCGCTTATTGATATGC – 3'		

Table 2. PCR conditions using ITS1 and 4 primers.

No.	Step	Temperature / °C	Time	Cycles No
1	Denaturation 1	94	5 min	1
2	Denaturation 2	94	30 s	25
3	Annealing	56	45 s	25
4	Extension	72	1 min	25
5	Final Extension	72	7 min	1

Omega. The phylogenetic tree construction used MEGA 11 with the protocol of maximum likelihood method (Kumar *et al*., 2016; Tamura *et al*., 2021; Ahmed and Abass, 2022).

Pathogenicity experiment

Pathogenicity tests on faba bean plants progressed in a fully controlled greenhouse environment. The greenhouse maintenance had a temperature range of 15 °C–20 °C and a relative humidity of 85%. Seeds from local varieties of faba bean plants underwent planting in pots with a diameter of 20–25 cm. The fungal treatments and a control group trials used 10 replications. The conidial suspension of the examined fungus preparation followed the method described by Rashid *et al.* (2016) and Ahmed and Abass (2022). Spores extraction using a haemocytometer from the agar surface acquired a 1×106 colony forming unit/mL concentration.

The obtained spore suspension came from flooding the Petri plate containing 7-day-old fungal growth on PDA with sterile distilled water. Within the greenhouse, the faba bean plants in pots received spraying with the respective conidial suspension of the fungus. Both treated plants and control samples maintenance comprised covering with transparent plastic bags that allow light to pass through. Securing these plastic covers were kept in place for 48 hours. After two weeks of post-inoculation, the disease incidence gained evaluation. It involved observing and assessing the symptoms of infection in the faba bean plants. A re-isolation of fungal pathogen followed to achieve Koch's postulates (Yu *et al*., 2022).

RESULTS AND DISCUSSION

Fungal isolation and description

The diagnosis of the fungus isolated from the faba bean (*V. faba* L.) plants initially referred to its phenotypic characteristics. Further confirming the identification of the fungal species led to molecular diagnostic studies using PCR amplification of DNA fragments from the internal transcribed spacer (ITS) region, a globally recognized molecular code. These experiments confirmed the presence of *Exserohilum rostratum*. This discovery was noteworthy as it marks the first documented reference of this fungus on faba bean plants in Iraq and worldwide.

Morphological characteristics

The color of the pure colony of the *E. rostratum* grown on PDA medium ranged from gray to blackish brown, with rough edges and a cottony appearance (Figures 1A, B). However, the colony back was dark brown, and the said colony reached the maximum of its growth (90 mm) after seven days of incubation at a temperature of 25 °C**.** The results also enunciated that the fungus produces longitudinal to slightly curved conidia, with a length of 18.3–23.31 × 6.67–11.6 µm, and the

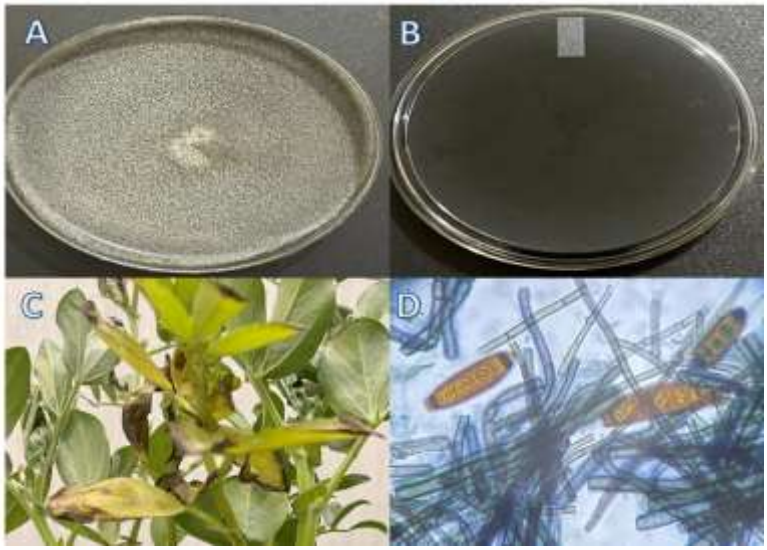

Figure 1. The colony morphology and microscope features of *E. rostratum* on a PDA medium. A) The top colony appearance, B) The reverse colony appearance, C) Pathological symptoms on Faba bean, and D) Conidia (40× magnification).

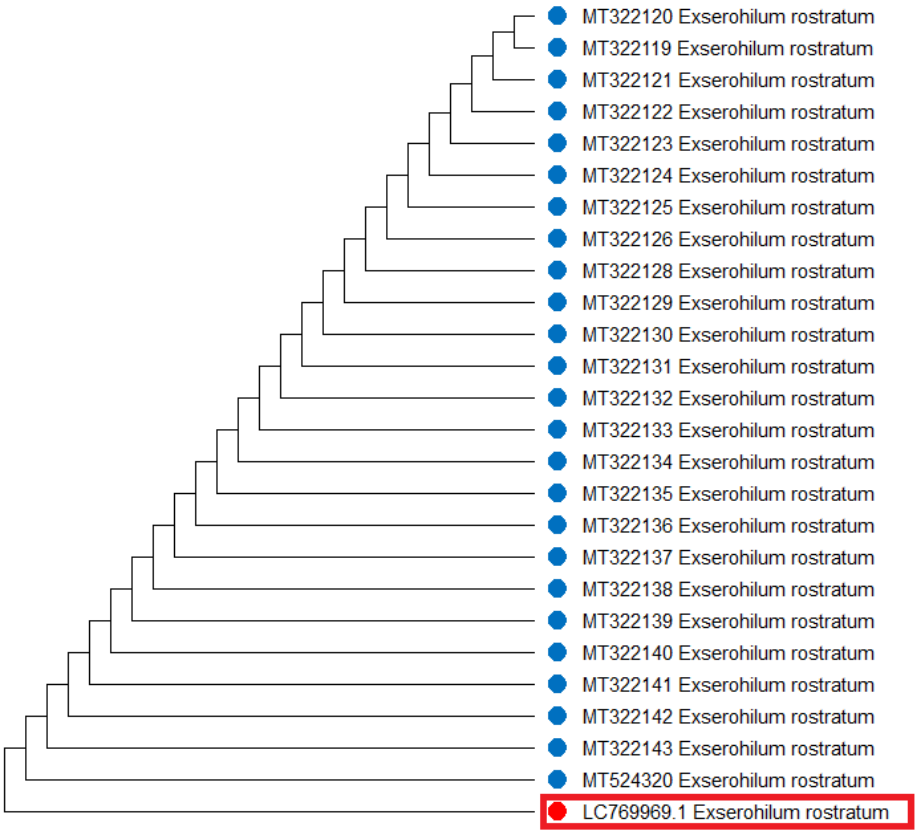

Figure 2. Phylogenetic tree constructed by the Neighbor-Joining method using ITS sequence for *E. rostratum* Iraqi isolate (LC769969.1) with the nearest 25 *E. rostratum* published in the GenBank.

transverse divisions were 3–4. The presented results about the diagnosis were analogous to previous findings (Sun *et al*., 2023; Tang *et al*., 2023).

Molecular identification and phylogenetic tests

For the molecular characterization of *E. rostratum*, scrutinizing the ITS region of ribosomal DNA (rDNA) used specific primers, namely, ITS1 and ITS4. This analysis revealed a noteworthy resemblance with the other fungi exhibiting similar phenotypic characteristics. The sequence data obtained, encompassing a range of 550 to 600 base pairs, displayed a remarkable similarity (99%) to the known fungus *E. rostratum* (represented by GenBank No. LC769969). The ITS sequence of *E. rostratum* isolates belonging to Iraq proceeded to deposit in NCBI under the accession number LC769969.

The phylogenetic subtree displays the branch length values demonstrating the relationship between the identified fungi and the reference fungi (Figure 2). Molecular identification techniques, also known for their high reliability, benefited the latest study. The results further revealed a substantial nucleotide similarity of 99% with recognized plant pathogenic fungi, which received support from the BLAST search. The ITS region encompasses the ribosomal RNA gene complex containing 16S, 5.8S, and 28S RNA subunits, widely accepted and employed for molecular identification in diverse plant pathogens (Abass, 2016). The analysis of phylogenetic results (based on the ITS gene sequence of *E. rostratum* and other nearest 25 hits obtained by NCBI-BLAST search results according to the Maximum likelihood method in MEGA 11) revealed that the Iraqi isolate of *E. rostratum* was close to the Indian isolate MT524320.1. The results obtained through a phylogenetic analysis align with the BLAST search conclusions.

In the latest study, the phenotypic and molecular diagnoses agree with findings from previous studies (Cardona and González, 2007; Farag, 2020). The observed phenotypic characteristics and the molecular analysis coincide with the outcomes reported in several other research investigations. The consistency in diagnosis across different studies strengthens the validity and reliability of the results gathered in the present study (Farag, 2020; He *et al*., 2023). The evolutionary distances computation used the Poisson correction method and was in the unit range of the number of amino acid substitutions per site. The evolutionary analyses ran in MEGA11.

The presented study had faba bean plants inoculated with a $1x10^6$ cfu/mL conidial suspension of *E. rostratum*, causing the onset of initial disease symptoms five days post-inoculation on young leaves. The disease symptoms presented round to oval white spots, each with a lesion size of approximately 0.2–0.5 cm. As the disease progressed, small spots on both leaves and stems merged, forming larger lesions with a distinctive black color, mirroring observed field symptoms on faba bean plants (Figure 1C).

The results revealed a severity level of 45% on the faba bean local cultivar plants in assessing disease severity at 14 days post-inoculation. In contrast, all the untreated plants (inoculated with dH_2O) remained healthy throughout the pathogenicity trial. Re-isolation of *Exserohilum rostratum* from necrotic tissues of treated faba bean plants enunciated reliable characteristics with the original *E. rostratum* isolate, confirming Koch's postulates. The presented study concluded the first documentation of *E. rostratum* as a potential pathogen causing aerial parts spot disease in faba bean plants in Iraq. The pertinent results were in harmony with various past studies, as they have reported *E. rostratum* as a virulent fungus on various economic plants, such as sun choke, rice, and beans (Farag, 2020; Khaekhum *et al.*, 2021; Kaboré *et al.*, 2022).

CONCLUSIONS

In the prominent study about the fungus *Exserohilum rostratum* on the faba bean (*V. faba* L.), the genetic analysis confirmed the identity of the fungal species, displaying a similarity of 99% (LC769969). The results also demonstrated a high level of pathogenicity,

and the examined symptoms were consistent with those observed in the fields of Basrah City, Iraq. It is worth noting that the recent research represents the first report in Iraq and globally of *E. rostratum* identified as a potential pathogen affecting the faba bean plants.

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