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The First Record of the Fungus *Fusarium incarnatum* (Desm.) Sacc., (1886) as a Potential Pathogen of Leaf Spot Disease on the Broad Bean *Vicia faba* L. in Iraq

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Abstract. Isolation of the fungus *Fusarium incarnatum* (Desm.) Sacc. was performed at the laboratories of the College of Agriculture at the University of Basrah from infected bean plants showing symptoms of fungal leaf. The fungus identity was revealed depending on phenotypic and microscopic examination, as well as a molecular technique have been applied using an ITS primer (Its1 and 4), the results of phylogenetic analysis with a similarity index of 98% according to NCBI –BLAST tool (LC769967). The pathogenicity was determined according to Koch's postulates, and severe symptoms were examined on the leaves of the broad bean plants during testing. This record is a significant as it is the first report of *F. incarnatum* causing spot symptoms on broad bean plants in Iraq and worldwide.

Keywords. Broad bean, *Fusarium incarnatum*, ITS, Phenotypic diagnosis, Molecular diagnosis.

1. Introduction

Legumes are an important source of protein and can serve as an alternative to animal protein. Broad beans are among the oldest plants cultivated in Mediterranean countries and Southeast Asia [1,2]. Faba beans (*Vicia faba* L.) are one of the oldest crops worldwide and rank as the third most important feed grain legume, which currently cultivated on a large scale in 58 countries. Faba beans have shown remarkable resilience to global warming and climate change, adapting well to various climatic conditions and thriving in diverse soil environments. Unfortunately, despite being a highly versatile and nutritious crop, some regions of the world remain unaware of its potential and benefits [3]. Faba beans are an ancient cool-season legume crop that relies on biological nitrogen fixation. They play a crucial role in global agriculture and human diets. Faba beans have a high nutritional value, surpassing peas (*Pisum sativum* L.) and other grain legumes in certain areas worldwide. In terms of cultivated area and production, faba beans rank third among grain legumes globally, following soybean (*Glycine max* Merr.) and peas [4,5].

The broad bean plant is susceptible to various diseases, including shoot system diseases that result in spots or blight on the leaves and stems. Severe symptoms of spot have been observed on the leaves and stems of broad bean plants in multiple areas of Basrah province. Therefore, the research aimed to isolate and diagnose the pathogen of these symptoms on the bean plant.

2. Materials and Methods

2.1. Sample Collection and Fungal Isolation

Symptomatic plant samples, including leaves and stems, were collected during the fourth quarter of 2022 and the first quarter of 2023 in Basrah province, Iraq. At the laboratory, the relevant samples were subjected to a series of procedures. First, they were washed using running water and then sterile distilled water. After that, the samples were cut into small pieces measuring 0.5-1 cm in length. To eliminate any fungi and bacteria present on the surface of the plant parts, they were washed for three minutes with a 5% NaOCl solution. Subsequently, the samples were once again washed with sterile distilled water to remove any traces of the sterilizing solution. The plant parts were then dried using filter paper. Next, Petri dishes (9 cm) containing the standard PDA culture medium was prepared. Four pieces of the plant parts were placed in each plate, and three plates were used for each portion of the plant. The Petri dishes were then incubated in an incubator at a temperature of 25 ± 2 °C for a period of 5-7 days, allowing the fungi to grow on the culture medium [6]. To obtain pure fungal isolates, the developing fungal colonies were purified. A portion of the fungus growing around the plant parts was taken using a cork borer that had been sterilized with alcohol and flame. The isolated portion was then transferred to Petri dishes containing the PDA culture medium. These plates were incubated under the same conditions as before to ensure the growth of pure fungal isolates.

2.2. Morphological Identification of Isolated Fungi

To document the morphological characteristics of the isolated fungi, a 5-mm diameter disc was aseptically extracted from the pure colonies after 5 days of growth. This disc was placed at the center of a Petri dish containing PDA (Potato Dextrose Agar) medium, and the dishes were incubated in an incubator. The fungi were then examined and their morphological features were recorded using both naked-eye observations and a binocular microscope. This involved observing and noting the form, size, margin, luster, transparency, and colony color of the isolated fungi. In addition, the developing fungi were examined under a light microscope to identify the color of the fungal filaments and spore-bearing structures (conidia). The shape, color, size, and other microscopic characteristics relevant to their classification were also documented. Detailed records of the phenotypic and morphological characteristics of the isolated and identified fungi were kept. To preserve the fungal cultures, discs were taken from the colony edges using a sterile ring conveyor and used to inoculate solid slanted culture media. These slants were then stored at 4 °C in a refrigerator until they were needed for subsequent experiments [7-9].

2.3. Molecular Identification of Pathogens

The pure mycelium isolates were utilized for diagnosis and classification based on their morphological and microscopic characteristics. Additionally, molecular diagnostic experiments were conducted using the global molecular code, specifically the internal transcribed spacer (ITS), in the polymerase chain reaction (PCR). The PCR aimed to amplify digested fragments of DNA from the fungal isolates. To initiate the experiments, sterile ring conveyor filler was used to obtain samples from each test tube containing pure fungal isolates. These samples were then inoculated onto Petri dishes containing solid culture medium (PDA) with three replicates for each fungal species. The Petri dishes were incubated in an incubator at a temperature of 25 ± 2 °C for a period of seven days. This allowed the fungal colonies to grow and be used for the subsequent extraction of genetic material, specifically DNA, which was required for the molecular diagnosis using PCR. The PCR was carried out on the extracted DNA from the fungal isolates, following the method outlined by [10, 11] for amplification.

The primer sequences used in the PCR can be found in Table (1), while the PCR conditions were illustrated in Table (2). The resulting PCR products were then subjected to sequencing, and the sequence outputs were submitted to the National Center of Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/) for BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search and homology analysis. The sequence of each species gene was finally deposited at NCBI as advised by [12].

Table 1. Primer sequences that adopted in PCR technology.

Primer	Primer sequence	Length of PCR product	Reference
ITS-1	5'- TCCGTAGGTGAACCTGCGG - 3'	550-600 (bp)	[13].
ITS-4	5'- TCCTCCGCTTATTGATATGC – 3'		

Table 2. PCR conditions using ITS1 and 4 primer.

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

2.4. Pathogenicity Experiment

Pathogenicity tests were conducted on faba bean plants in a greenhouse environment with a temperature range of 15-20 °C and 85% relative humidity. The seeds of the relevant faba bean plants were planted in pots with a diameter of 20-25 cm. Each fungal species, along with a control group, was tested with ten replicates using faba bean plant pots.

Following the method described by [14].a conidial suspension for each fungus was prepared. This involved extracting spores (scraped from the surface of agar) at a concentration of (1×10^6 cfu /ml) using a haemocytometer. The spore suspension was obtained by flooding the Petri plate containing the fungal growth on PDA (7 days old) with sterile distilled water. The faba bean plants in the pots inside the greenhouse were then sprayed with the conidial suspension of the respective fungi. To maintain moisture and prevent leaf drying, the treated plants and control samples were covered with transparent plastic bags that allowed light to pass through. This coverage was maintained for 48 hours. The disease incidence was evaluated two weeks after inoculation, allowing for the observation and assessment of any symptoms or signs of infection on the faba bean plants.

3. Result and Discussion

3.1. Fungal Isolation and Description

The fungus isolated from the faba bean plant was diagnosed based on its phenotypic characteristics. Additionally, the phenotypic and microscopic diagnosis of the fungal species was confirmed through molecular diagnostic experiments using the global molecular code of the ITS (internal transcribed spacer) in the PCR (polymerase chain reaction) to amplify DNA fragments. The diagnosis revealed the presence of the fungus *F. incarnatum*. This finding is significant as it represents the first recorded instance of this fungus on the faba bean plant in both Iraq and globally.

3.2. Morphological Characteristics

Figure 1 show that the color of the pure colony grown on PDA medium ranged from creamy to yellowish-brown, with rough edges and a cottony appearance. The reverse side of the colony appeared creamy to light brown, and the colony filled the plate within 4-5 days. The fungus was found to produce three types of conidia spores: Macroconidia, which measured 16.65-23.31 x 3.34-3.99 µm, Microconidia, with dimensions of 8.32-13.32 x 2.66-3.98 µm, and Chlamydospores, which had dimensions of 9.98-10.82 x 6.67-8.32 µm (Fig. 1 D). The findings of the diagnosis is in a good agreement with previous studies [15,16].

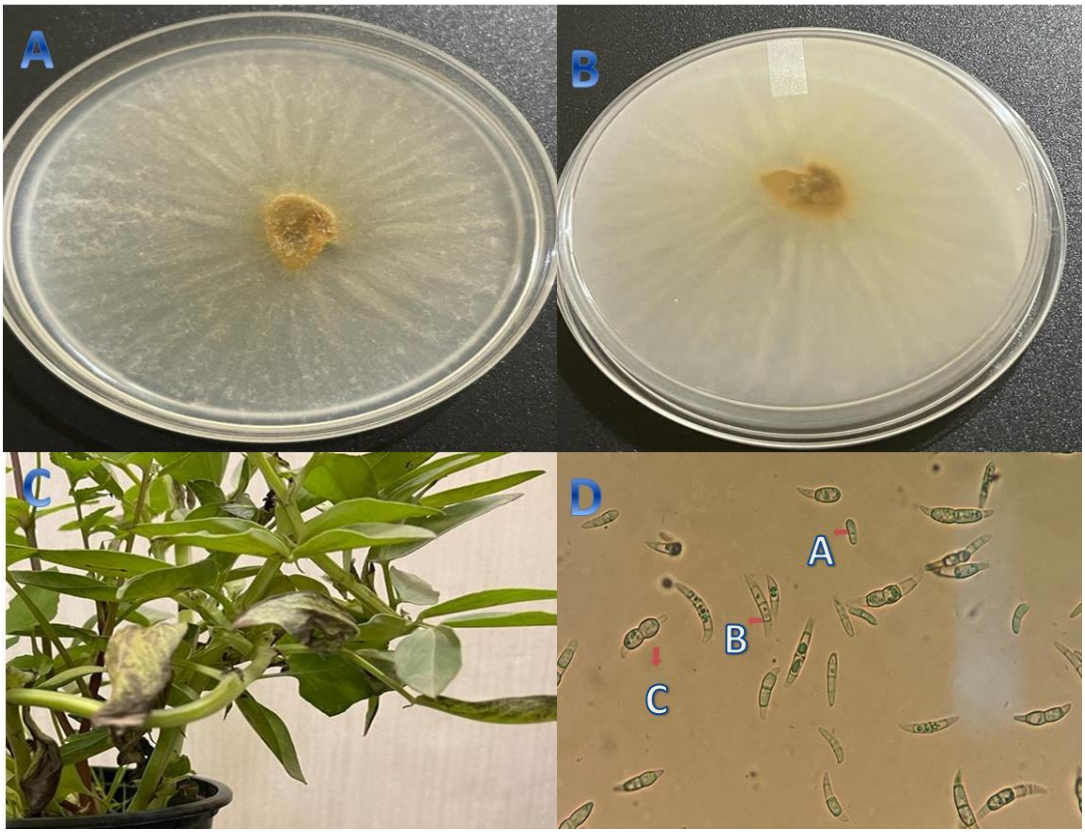

Figure 1. Phenotypic features of *F. incarnatum*. (A) The frontal appearance of the colony (B) the dorsal appearance of the colony. (C) Pathological symptoms on broad bean (D) Conidia (40× magnification) A- Microconidia, B- Macroconidia, C- Chlamydospores.

3.3. Molecular Identification and Phylogenetic Tests

The molecular characterization of *F. incarnatum* involved analyzing the ITS region of ribosomal DNA (rDNA) using ITS1 and ITS4 primers. This analysis revealed a significant similarity to other fungi that share similar phenotypic characteristics. The extracted sequence data, spanning 550 to 600 base pairs, showed a 98% identity with the known fungus *F. incarnatum* (represented by GenBank No. LC769967).

Figure 2 displays the branch length values in a phylogenetic subtree, illustrating the relationship between the identified fungi and reference fungi. Molecular identification techniques, which are highly reliable, were employed in this study. Our findings indicated a high degree of nucleotide similarity (98%) with recognized plant pathogenic fungi, and this was further confirmed through the results of the BLAST search. The ITS region, encompassing the ribosomal RNA gene complex consisting of 16S, 5.8S, and 28S RNA subunits, is widely accepted and utilized for molecular identification in various plant pathogens [17].The phenotypic and molecular diagnosis were consistent with several studies[18,19].

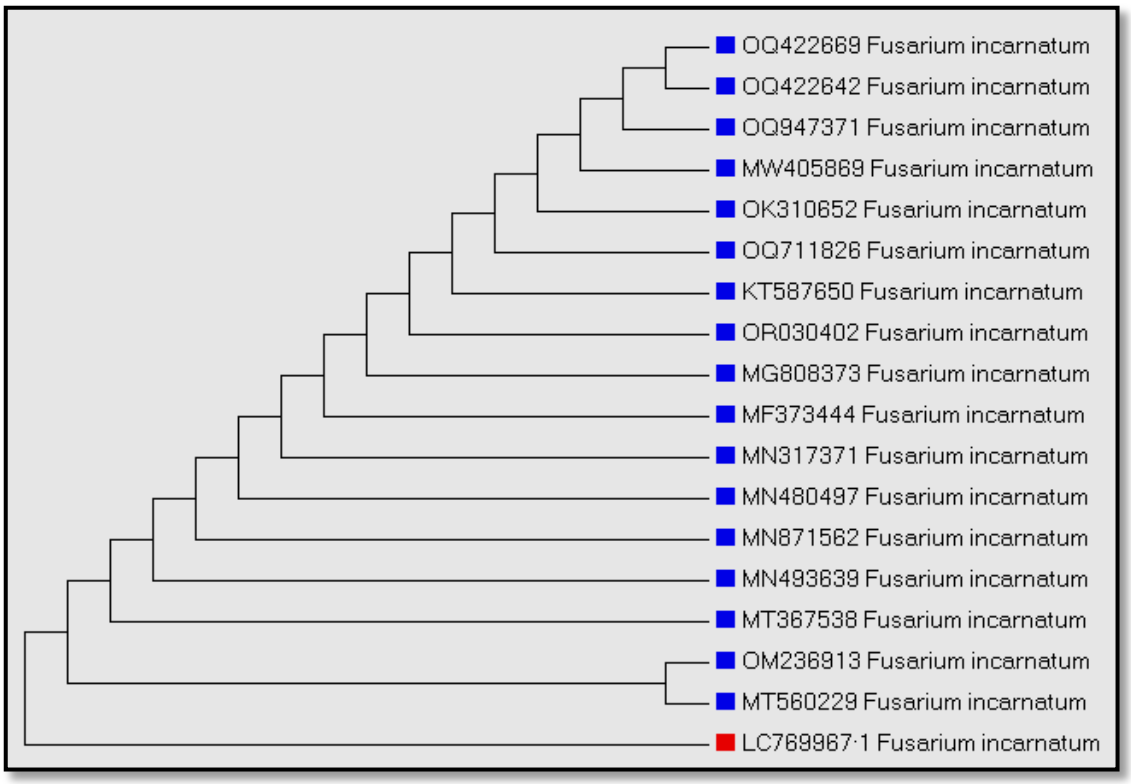

Figure 2. The phylogenetic tree generated by the Neighbor-Joining method for the genetic affinity of *F. incarnatum* in comparison with the standard sequences of nitrogenous bases in the NCBI/GenBank.

Several studies have confirmed that *F. incarnatum* is the causative agent of plant leaves spot disease [19,20].This study represents the first recorded case of *F. incarnatum* causing spots on broad bean plants in Iraq and worldwide. Further studies are necessary to identify the fungal genera that infect bean sprouts and explore ways to mitigate the damage caused by these fungi on bean plants.

Conclusions

In the current paper, the fungal species of *F. incarnatum* was isolated from symptomatic leaves of broad bean with leaf spot symptoms collected from Basrah different fields. Both morphometric and microscopic analysis were performed to identify the pathogen, followed by a molecular analysis using a universal primers of ITS, the genetic results analysis revealed the identity of fungal species with a similarity percent of 98% (LC769967). The Koch's postulates were done to reveal the potential pathogenicity of *F. incarnatum* on faba bean leaves, the results approved the high level of pathogenicity, additionally the symptoms were identical to what were examined at Basrah's fields. It's noteworthy that this paper is the first of its kind in Iraq and worldwide as a potential pathogen on broad bean.

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