First Report of *Alternaria burnsii* as a Foliar Pathogen of Faba bean in Iraq

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Abstract

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The current study highlighted the potential pathogenicity of casual fungus that causes leaf, stem and pod spot disease in faba bean. The symptomatic leaves and pods of faba bean were collected from different areas around Basrah city, south of Iraq. The isolation procedure was performed on PDA plates, followed by morphometric and microscopic identification. Briefly, a superficial white/orange to a creamy colour mycelium was observed with a circular entire edge and a flossy to smooth velvety morphology. The conidia were oval to oblong with 3-6 transverse septa, producing a set of 4-8 long-chain conidia. Molecular analysis of the ITS sequence confirmed the identity of the fungus; the fungus formed a distinct subclade within the *Alternaria burnsii* clade, and the ITS sequence was submitted to the NCBI under the accession number LC769966.1. Pathogenicity tests confirmed the virulence of *A. burnsii* on broad bean leaves under greenhouse conditions. First, disease symptoms were examined on young broad bean leaves five days' post inoculation. White necrotic spots appeared on the aerial parts of the broad bean, and each spot enlarged and coalesced to form necrotic lesions distinguished by black colour. The findings of this study revealed the pathogenicity of *A. burnsii* for the first time in the aerial parts of broad beans in Iraq.

Keywords: Faba bean, necrotic spot, molecular diagnosis, Iraq, *Alternaria burnsii*.

Introduction

Faba bean crop is among the oldest field crops in the world and rank third among the most important legume grains after soybean, *Glycine max* L. and pea, *Pisum sativum* L. (Mohammed, 2023; Singh *et al.*, 2013). The scientific name of the faba bean is *Vicia faba* L., which belongs to the family Fabaceae and is known with different names worldwide, such as broad bean, Horse bean, Windsor bean and many others (Mohammed, 2023). Faba bean plants are cultivated in more than 70 countries worldwide, including Iraq (Atab *et al.*, 2023; FAO, 2021; Merga *et al.*, 2019). The faba bean crop is considered a traditional food in the Mediterranean region and is cultivated mainly for its pods, which are rich in protein and approximately 35% seed dry weight. In addition to healthy compounds such as phenols, chlorophyll, carotenoids and vitamins (Kumar *et al.*, 2022).

Faba bean plants are susceptible to infection with several fungal pathogens which lead to a significant yield loss in both quality and quantity (Plūduma-Pauniņa *et al.*, 2019), among these diseases, those that attack the aerial part are considered the most important, such as chocolate spot (caused by fungal pathogens *Botrytis* spp.: *Botrytis fabae*, *B. cinerea* and *B. fabiopsis*) (Bankina *et al.*, 2017); *Ashochyta* blight (caused by fungal pathogen: *Didymella fabae*); *Cercospora* leaf spot (caused by fungal pathogen: *Cercospora zonata*) (Stoddard *et al*., 2010); rust (caused by fungal pathogen: *Uromyces viciae-fabae*); downy mildew (caused by fungal pathogen : *Peronospora viciae* f.sp. *fabae*) (Juroszek & Tiedemann, 2011); and *Stemphylium* leaf blight (caused by the fungal pathogens *S. botryosum*, *S. eturmiunum* and *S. vesicarium*) (Vaghefi *et al.*, 2020). However, the fungal pathogen *Alternaria* spp. is considered one of the most economically important pathogens of faba bean aerial parts, causing significant losses; this fungus has been known as *Alternaria* leaf spot and has become a predominant pathogen during recent decades (Bankina *et al.*, 2021). Several species, including *A. alternata*, *A. tenuissima*, *A. angustiovoidea* and others, have been reported as causal agents of Alternaria leaf spot disease (Bankina *et al*., 2021; Coca-Morante & Mamani-Álvarez, 2012; Konjengbam *et al.*, 2023; Yaser & Abass, 2022).

The fungal species of the genus *Alternaria* are among the most abundant fungi in different niches and environments (Hafez *et al.*, 2022; Yan *et al.*, 2022), some are common harmless saprophytes in soil, air and many other habitats; others are ubiquitous plant pathogens that cause serious diseases on economically important crops, attacking stems, leaves and fruits (Dettman *et al.*, 2023; Gou *et al.*, 2023; Lawrence *et al.*, 2013; Razak & Abass, 2021). The *Alternaria* genus contains more than 700 different species; 400 of these species are well classified and accepted into 28 sections, and only 100 species have been genetically identified (Ahmadpour *et al.*, 2021; Li *et al.*, 2023; Wijayawardene *et al.*, 2022). Additionally, this genus is classified within the family Pleosporaceae and the order Pleosporales, while the class is Dothideomycetes (Li *et al.*, 2022). *Alternaria* species are characterized by several unique features, such as micro- to macronematous, branched to unbranched conidiophores, discrete to integrated, mono- to polytretic conidiogenous cells, solitary to catentae, straight to curved, smooth or verrucose and median brown to dark brown conidia to rounded/narrowly beaked tips (Jayawardena *et al.*, 2019; Lawrence *et al.*, 2016; Li *et al.*, 2023). Regarding the species *Alternaria burnsii*, very few studies have focused on this pathogen because of the limited number of economically important plant families, such as Rhizophoraceae, Menispermaceae, Malvaceae, Poaceae and Amaranthaceae (Woudenberg *et al.*, 2015); thus, this

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pathogen has been reported to cause cumin blight (Aziz *et al.*, 2021; Shekhawat *et al.*, 2013; Özer & Bayraktar 2015), leaf spot of date palm and wheat (Al-Nadabi *et al.,* 2018), leaf blight of onion (Htun *et al*., 2022), and leaf spot of tomato (Dominique *et al.*, 2022). There are no reports regarding the infection of Broad bean aerial parts with *A. burnsii*, accordingly, this is the first report of this fungus causing disease on broad bean plants. Thus, the main objective of this study was to isolate and characterize the fungal pathogen *A. burnsii* via morphometric and molecular tools. A further objective was to apply Koch's postulates to reveal the pathogenicity of this fungus on faba bean plants.

Materials and Methods

Collection of samples

An extensive field survey was conducted between the fourth quarter of 2022 and the first quarter of 2023 at different fields in the Basrah governorate. Symptomic aerial parts of the faba bean plants showing severe leaf and pod spot symptoms were collected during the 2022-2023 season and brought to the laboratory and kept at 4°C for subsequent investigation.

Isolation of fungal pathogens

Briefly, the symptomatic aerial parts (leaves and pods) of faba bean plants were surface sterilized with 70% ethanol for one min, washed three times in distilled water and subsequently dried on sterile Whatman filter paper No. 1. The symptomatic spot plant parts were cut from their spot margins into small pieces of 2 mm in diameter, plated on potato dextrose agar (PDA) plates supplemented with 250 µg/L chloramphenicol and incubated at 25±1°C for a period of 5-7 days to allow fungal pathogen growth.

Morphometric and microscopic characterization of *A. burnsii*

The fungal pathogen was purified on PDA plates by a single spore technique and incubated for 7 days at 25±1°C to examine colony morphology and growth and mycelium pigmentation. Further light microscopy (Olympus BX51, Tokyo, Japan) was used to observe the shape, size and size of the conidia by measuring 100 conidia (Ahmed & Abass, 2022).

Extraction of fungal DNA

Genomic DNA was extracted from a pure colony of *A. burnsii* by using Fungal Mini Kit provided from Korean Company Favorgen Biotech Corp, following the manufacturer's instructions. Subsequently, the DNA concentration and purity were measured using a NanoDrop, Thermo Fisher spectrophotometer.

DNA amplification

The DNA amplification of the *A. burnsii* genomic DNA was made by the application of ITS (internal transcribed spacer) primers, forward ITS1 primer: 5'-TCCGTAGGTGAACCTGCGG-3' and reverse ITS4 primer: 5'-TCCTCCGCTTATTGATATGC-3' (White *et al.*, 1990). PCR was conducted with a final concentration of 25 µL using 9 µL of ddH_2O, 1 µL of both forward and reverse primers, 1.5 µL of genomic DNA and 12.5 µL of master mix. The PCR thermorecycling procedure was as follows: initial denaturation at 95°C for 5 min in one cycle; denaturation at 95°C for 30 seconds; annealing at 53°C for 2 min; extension at 72°C for 30 seconds for 35 cycles; and a final extension at 72°C for 7 min. PCR efficiency was evaluated by running the amplicon DNA fragments via 1.5% agarose gel electrophoresis.

Sequencing and phylogenetic analysis

The PCR products were sequenced by Macrogen Company-South Korea. The *A. burnsii* gene sequence was deposited in GenBank under the accession number LC769966.1 and analysed using the BLAST search tool at NCBI. Similar sequences were downloaded from the GenBank database and aligned using Clustal Omega. A phylogenetic tree was constructed using MEGA 11 with the maximum likelihood method (Kumar *et al.*, 2016; Tamura *et al.*, 2021). The percentage of replicate trees in which the associated taxa clustered together was analysed using the bootstrap test (1000 replicates), and the evolutionary distances were computed using the Poisson correction method and are expressed in units of the number of amino acid substitutions per site (Jones *et al.*, 1992).

Pathogenicity experiment

The pathogenicity test was performed in a controlled greenhouse at a temperature of 25±2°C and a relative humidity of 85%. The seeds of a local faba bean variety were sterilized with 75% ethanol and used for this test. Seeding was performed at 10 seeds/20 cm diameter pot in sterilised vermiculite and soil at the rate of 3:1, after which the plants were watered and monitored daily. The inoculum of *A. burnsii* was prepared according to Rashid *et al.* (2016) and Razak & Abass (2021) by plating a fungal disc of pathogen growth (hyphae and conidia) on a PDA plate and incubated at 25±1°C for 7 days. Subsequently, the conidia were collected in ddH_2O and three drops of Tween 80 and adjusted to 1×10^6 CFU/mL using a hemacytometer. Ninety days after sowing, the faba bean plants were inoculated with an *A. burnsii* spore suspension at 1×10^6 cfu/mL, and dH_2O was used as a control treatment (Razak & Abass, 2021). The treated plants were covered with plastic bags for two days to maintain a high level of humidity. At 14, 28 and 42 days postinoculation, disease progress was monitored by visualising the disease symptoms and evaluating the conidia of the fungal pathogen via light microscopy. Disease severity was assessed according to the Simko & Piepho (2012) 0-9 scale as follows: 0= no symptoms, 1= 1-3 spots on the whole plant, 2= 4-6 spots on the whole plant, 3= 10% of the whole plant covered by spots, 4= 11-25% of the whole plant covered by spots, 5= 26-50% of the whole plant covered by spots, 6= 51-75% of the whole plant covered by spots, 7= More than 75% of the whole plant covered by spots, 8= 100% of the whole plant covered by spots, 9= Plant dead.

The disease severity index (DSI) was evaluated according to the equation of Mckinney (1923), and the pathogen was reisolated from infected plant parts to apply Koch's postulates.

$$\text{Disease Severity Index}=\frac{\text{Scale score}\times\text{Frequency}}{\text{Total number of units}\times\text{Maximum score}}\times 100$$

Results and Discussion

Disease symptoms in the field

Disease symptoms on faba bean aerial parts were observed during 2022-2023 in several fields in Basrah region. Disease symptoms developed progressively when favourable conditions prevailed, such as cold and humid weather, and led to the spread of disease throughout the stems, leaves and pods. Symptoms appear first as small, necrotic circular spots on the aerial parts (stem and leaves) with white colour in the centre and brown to dark brown margins, and then spots enlarge in size, coalesce with each other and become thick and dark brown to black in colour (Figure 1). When the environmental conditions are favourable for the disease, whole broad bean leaves and pods are covered with necrotic spots, which can lead to the death of infected plants. These findings are in agreement with those of several studies that confirmed the virulence of *A. burnsii* on economically important plants, such as date palm, wheat, cumin, onion and tomato (Al-Nadabi *et al.*, 2018; Aziz *et al.*, 2021; Dominique *et al.*, 2022; Htun *et al.*, 2022).

Figure 1. Symptoms of faba bean spot disease caused by *A. burnsii* in the Basra region. (A) Leaf and stem spot disease symptoms, (B) Symptoms on pods.

Morphometric and microscopic characteristics of *A. burnsii*

A. burnsii exhibited maximum growth (90 mm) on PDA plates, 7 days after incubation at 25±1°C. The fungal colonies that grew on PDA media were white in colour, whereas the margins of the colonies were white with a circular entire edge and a flossy to smooth velvety morphology, while at the bottom of the plate a yellowish white or yellowish-brown colour was observed at the growth centre (Figure 2, A-B). *A. burnsii* conidiophores were branched, erect and straight, irregularly bent and geniculate, and produced a set of 4-8 conidia. Conidia were oval to oblong in shape with 3-6 transverse septa and 10 µm × 45.5 µM in size (Figure 2 C). All the morphometric and microscopic features of *A. burnsii* are in accordance with the description made by Simmons (2007).

Molecular characterization

The PCR amplification of the internally transcribed spacer (ITS) gene sequences of the fungus *A. burnsii* amplified a specific DNA fragment of approximately 530 bp. This sequence was subjected to a NCBI-BLAST similarity search and multiple sequence alignment via the application of Clustal Omega. The results obtained revealed a similarity rate of 100% with the sequence of *A. burnsii* Indian isolate (OL304934.1), which was subsequently submitted to GenBank under accession number LC769966.1. The phylogenetic analysis was subsequently performed based on the ITS gene sequence of *A. burnsii* and 15 others nearest *Alternaria* species obtained by BLAST search according to the maximum likelihood method in MEGA11, and the results revealed that the Iraqi isolate of *A. burnsii* was similar to the Indian isolate (Figure 3). The phylogenetic analysis results were in accordance with the BLAST search results. The efficiency of the ITS gene in the molecular identification of *A. burnsii* revealed by the present study, was consistent with the findings of many previous reports (Singh *et al.,* 2016; 2018; Lan *et al.,* 2023).

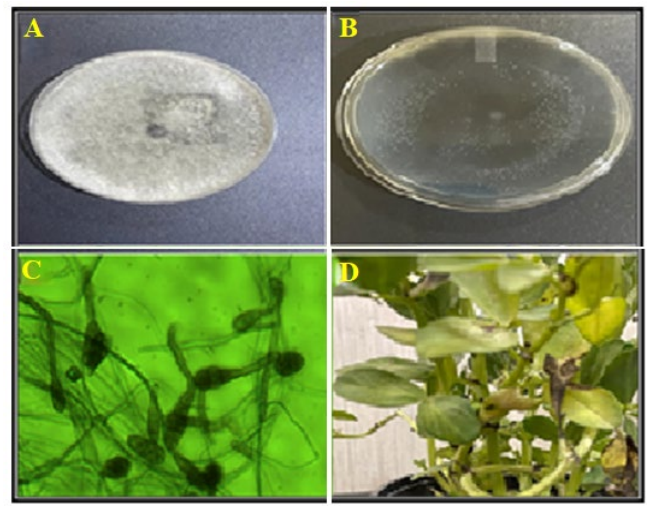

Figure 2. *Alternaria burnsii* colony morphology and microscopic features on PDA plates and symptoms produced on faba bean. (A) colony appearance, (B) Bottom colony appearance, (C) Conidia (40x magnification), (D) Disease symptoms on the faba bean aerial parts in the pathegencity test.

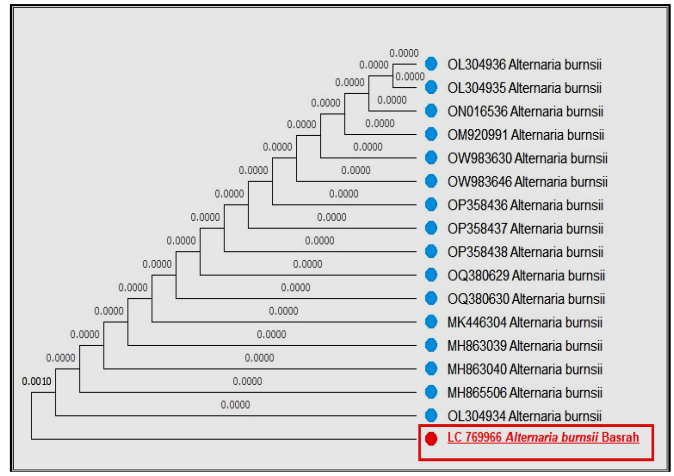

Figure 3. Phylogenetic tree constructed by the neighbor–joining method using the ITS sequence of the *Alternaria burnsii* Basrah isolate (LC769966.1) with the nearest *A. burnsii* published by GenBank.

Pathogenicity experiment

An *A. burnsii* conidial suspension of 1×10^6 CFU/mL was used for faba bean plant inoculation. The first disease symptoms developed five days after inoculation on young leaves as round to oval white spots with a lesion size of approximately 0.2-0.5 cm. After disease symptoms developed, the small black spots coalesced with each other on both leaves and stems, which was similar to what was observed on faba bean in the field (Figures 1, B and 2, D). The disease severity on the faba bean plants local variety 14, 28 and 42 days after inoculation was 30, 45 and 65%, respectively. All the untreated plants (inoculated with dH_2O) remained healthy during the pathogenicity trial. *A. burnsii* was reisolated from necrotic tissues of inoculated faba bean plants, and the results were consistent with those of the *A. burnsii* isolate, thus Koch's postulates were confirmed.

The fungus *A. burnsii* has been reported in numerous studies as a virulent pathogen on many plants, such as date palm, wheat, cumin and tomato (Al-Nadabi *et al.*, 2018, Aziz *et al.*, 2021, Dominique *et al*., 2022). This study is the first report of *A. burnsii* as a potential pathogen on faba bean plants causing foliage spot disease. The importance of *A. burnsii* as a potential plant pathogen could be attributed to many factors, including seed-transmissibility and its remarkable survival on seeds and plants (Khare *et al.*, 2014; Sharma *et al.*, 2013; Singh *et al.*, 2016).

To the best of our knowledge, this study is the first report of *A. burnsii* as a causative agent of faba bean spot disease in Iraq. The molecular identification revealed the identity of *A. burnsii* based on the ITS gene sequence, deposited in NCBI GenBank under accession number LC769966. A significant increase in disease severity was observed in faba bean plants 42 days after *A. burnii* inoculation. However, additional studies are needed to determine the importance of disease complexes with other *Alternaria* species in causing severe faba bean diseases.

الملخص

عوفي، بيداء غازي، يحيى عاشور صالح ومحمد حمزة عباس. 2025. التسجيل الأول للفطر *Alternaria burnsii* كممرض على الأجزاء الهوائية لنبات الفول/الباقلاء في العراق. مجلة وقاية النبات العربية، 43(3): 304–309. https://doi.org/10.22268/AJPP-001330

هدفت الدراسة الحالية إلى تحديد المسبب الممرض لتبقع أوراق وسوق وقرون نبات الفول/الباقلاء الذي ظهرت أعراضه في مناطق مختلفة من محافظة البصرة/العراق، حيث تمّ جمع عينات نباتية تمثل الأجزاء الهوائية المصابة بالفطر الممرض من نباتات الفول/الباقلاء، وأجريت عملية العزل على الوسط الزرعي PDA. تمّ تحديد هوية الفطر الممرض بناءً للخصائص المظهرية والمجهرية والتي بينت أنه النوع *Alternaria burnsii*. تمّ تأكيد التشخيص اعتماداً على التشخيص الجزيئي باستعمال بادئات ITS والتي سجّلت تتابعات قواعده الآزوتية في بنك الجينات تحت الرقم LC769966 وتطابقت مع الفطر *A. burnsii* بنسبة تشابه بلغت 100%. كما أثبتت نتائج اختبار الإمراضية في ظروف البيت الزجاجي مقدرة عزلة الفطر *A. burnsii* على إحداث الأعراض المرضية على نباتات الفول/الباقلاء المعداة بالفطر، حيث ظهرت الأعراض الأولى بعد خمسة أيام من العدوى، لتشتدّ الأعراض على المجموع الخضري مسجلة شدّة إصابة بلغت 30، 45 و 65% بعد 14، 28 و 42 يوماً، على التوالي، من العدوى الصناعية بالفطر *A. burnsii*. تعدّ الدراسة الحالية أول تسجيل للفطر *A. burnsii* كممرض على نبات الفول/الباقلاء في العراق.

كلمات مفتاحية: نبات الفول/الباقلاء، مرض التبقع، التشخيص الجزيئي، العراق، *Alternaria burnsii*.

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