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Species diversity and molecular characterisation study of different *Alternaria* species associated with Faba bean (*Vicia faba* L.) aerial parts diseases in Basrah

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ABSTRACT

Several spots and necrotic symptoms fungi-caused in the aerial parts of the Faba bean (*Vicia faba* L.) are spreading and causing painful economic losses for farmers in Basrah Province. Fungi were isolated from severely infected faba bean leaves and stems with leaf spots and necrosis from farms in the Shaat-Al-Arab and Abu-Alkhaseeb regions. Morpho-cultural examinations on media and molecular analysis using internal transcribed spacer (ITS) primers and sequence and phylogenetic analysis were carried out to identify fungi of spots-causative, and tested for their pathogenicity. Morpho-cultural examinations showed that isolated fungi typified diverse species of *Alternaria*. While molecular and phylogenetic analyses revealed the identities of eight different *Alternaria* species: *A. alstroemeriae*, *A. arborescens*, *A. chlamydospora*, *A. concatenta*, *A. gaisen*, *A. infectoria*, *A. porri* and *A. terricola*. Each ITS sequence was deposited at NCBI and submitted with a gene accession number. Pathogenicity tests revealed that all *Alternaria* species were able to induce disease symptoms on the local sensitive variety of Faba bean under greenhouse conditions; these disease symptoms were similar to those reported in the field. This is the first report of several *Alternaria* species causing Faba bean aerial part spot and necrosis disease in Basrah/ Iraq. Further studies are needed to better understand the disease complexity among these species of pathogens and to identify the best measures to control the disease.

دراسة التنوع والتشخيص الجزيئي لأنواع مختلفة من الفطر *Alternaria* spp. المرتبطة بأمراض الأجزاء الهوائية لنبات الباقلاء *Vicia faba* L. في محافظة البصرة

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الخلاصة:

تنتشر أعراض التبقع والتنخر النسيجي المتسببة عن الفطريات على الأجزاء الهوائية لنبات الباقلاء (*Vicia faba* L.) في محافظ ةالبصرة، مسببةً خسائر إقتصادية ملحوظة، حيث تم عزل عديد من الفطريات من أوراق وسيقان نبات الباقلاء المصابة بشدة بالتبقع من مزارع في منطقتي شط العرب وأبي الخصيب. تم إجراء فحوصات مظهرية ومجهرية على أوساط النمو الخاصة، فضلاً عن التشخيص الجزيئي باستخدام بادئات من نوع ITS كما أُجريت تحليلات التتابعات للقواعد النايتروجينية والنسب التطوري لتحديد الفطريات المسببة للتبقع، واختبار مدى مقدرتها على إحداث المرض. أظهرت الفحوصات المظهرية والمجهرية أن الفطريات المعزولة تنتمي إلى أنواع مختلفة من جنس *Alternaria* spp. بينما كشفت التحليلات الجزيئية عن وجود ثمانية أنواع مختلفة كانت *Alternaria alstroemeriae* و *A. arborescens* و *A. chlamydospora* و *A. concatenta* و *A. infectoria* و *A. gaisen* و *A. porri* و *A. terricola* ، كما تم إيداع كل تسلسل ITS في قاعدة بيانات NCBI مع رقم دخول جيني خاص. وأظهرت اختبارات الإمراضية أن جميع أنواع الفطر *Alternaria* spp. قادرة على إحداث أعراض المرض على صنف الباقلاء المحلي الحساس في ظروف البيت الزجاجي، وكانت أعراض المرض مشابهة لتلك التي لوحظت في الحقل وتُعد هذه الدراسة الأولى من نوعها التي أشارت إلى عزل وتشخيص عن أنواع متعددة تابعة للجنس *Alternaria* كمسببات لمرض التبقع على الأجزاء الهوائية لنبات الباقلاء في محافظة البصرة / العراق، مع تأشير الحاجة إلى المزيد من الدراسات لفهم تعقيد هذا المرض بشكل أفضل بين هذه الأنواع المختلفة من الممرضات، ولتحديد أفضل السبل لمكافحته.

INTRODUCTION

Faba bean (*Vicia faba* L.) is considered one of the oldest field crops in the world and is highly important as a legume grain after soybean (*Glycine max* L.) and pea (*Pisum sativum* L.) (Mohamed 2023). The broad bean (*Vicia faba* L.) belongs to the family Fabaceae and is known by different names, such as Broad bean, Horse bean, and Fava bean (Atab *et al*. 2023). Faba bean plants are cultivated in more than 70 countries worldwide. In Iraq, the cultivation of Faba bean has spread in recent years; in 2020, the total cultivated area was 2700 hectares, with a total production of 41,000 tons (Merga *et al*. 2019). The significant importance of Faba broad bean is attributed to several factors, including the nutritional value of its grains, which are rich in protein (35% of dry matter), starch, phenols, chlorophyll, carotenoids and vitamins (Alrawi *et al*. 2023). Several important diseases have been detected in Faba bean plants caused by fungi, bacteria, viruses and nematodes; among these, fungal pathogens are reported to cause serious economic losses, most commonly aerial diseases such as *Ashochyta* blight, chocolate spot, *Cercospora* leaf spot, downy mildew and rust, *Stemphylium* leaf blight and *Alternaria* leaf and stem spot, *Fusarium* leaf spot

(Stoddard *et al*. 2010; Vaghefi *et al*. 2020; Bankina *et al*. 2021; Ofi *et al*. 2023). The *Alternaria* genus is considered one of the most abundant genera in the world and can be isolated from different environmental substrates (Woudenberg *et al*. 2013; Hafez *et al*. 2022; Yan *et al*. 2022). Different activities have been reported for the species *Alternaria*. Some species are characterized by harmless saprophytes in soil and air, while others are active endophytes in many plant families, and many other *Alternaria* species cause serious diseases in important agricultural crops (Razak and Abass 2021; Dettman *et al*. 2023; Gou *et al*. 2023). Hundreds of different plant families are subjected to *Alternaria* infection, which occurs on a wide range of plant parts, including stems, leaves, pods and seeds, in addition to postharvest losses via food spoilage and dangerous mycotoxin synthesis (Woudenberg *et al*. 2013; Aichinger *et al*. 2021).

The first description of *Alternaria* Nees was in 1816, with *A. tenuis* Nees as the type (Nees von Esenbeck 1816); since then, approximately 1,100 species have been described; among these species, 400 species are well classified, and only 100 species have been genetically identified (Ahmadpour *et al*. 2021; Wijayawardene *et al*. 2022; Li *et al*. 2023). The *Alternaria* genus belongs to the family Pleosporaceae and the order Pleosporales and to the class Dothideomycetes (Li *et al*. 2022).

Several prominent species have been isolated and described as true plant pathogens that cause leaf spot diseases within the genus *Alternaria* on a vast array of economic plants, including *A. alternata*, *A. tenuissima*, *A. angustiovoidea*, *A. arborescens*, *A. burnsii* and others (Coca-Morante and Mamani 2012; Bankina *et al*. 2021; Razak and Abass 2021; Htun *et al*. 2022; Yaser and Abass 2022). Here, the present study aimed to identify the species of the *Alternaria* genus on morphological and molecular levels; and examine their pathogenicity on the aerial parts of Faba beans, which are among seven novel pathogenic species in Iraq.

MATERIALS AND METHODS

Sample collection and isolation of fungi

Leaves and stems of symptomatic plants were collected from different fields at Shaat-Al-Arab and Abu-Alkhaseeb in Basrah Province during at 2022-2023 season, and disease symptoms were recorded and documented. The symptomatic plant materials were placed in an envelope and stored at 4°C for further investigation. In brief, the diseased plant materials were rinsed with distilled water dH_2O, surface sterilized with ethanol (75%) for one minute, washed with dH_2O

three times and dried on sterile Whatman No. 1 filter paper. Subsequently, the diseased plant material spot margin was cut into a segment of 0.5 cm, plated on a Petri dish containing potato dextrose agar (PDA; supplied by Himedia; India) at the level of 39 g/L, and supplemented with chloramphenicol (250 µg/L) and incubated at 25 ± 1°C for a period of 7 days to allow the growth of the fungal pathogen. Five segments of disease materials were placed in each dish (Abass 2016).

Morphometric and microscopic identification of fungi

The fungal isolates were purified on PDA plates to explore colony morphology, growth and mycelium pigmentation. A 0.5 cm margin growth layer of each fungal colony was removed, and the colonies were cultured on a new PDA plate for 7 days at an incubation temperature of 25 ± 1°C and a 14:10 light/dark cycle. A light microscope (Olympus BX51, Tokyo, Japan) was used to record the shape, size of the conidia by measuring 100 conidia (Ahmed and Abass 2022), and their photos were taken by Saxon 3 MegaPixel Camera. The description of each fungus was recorded based on Simmons (2007).

Molecular identification of fungi

DNA extraction and amplification

The fungal total genomic DNA was extracted from 30 mg of fungal growth medium according to the manufacturer's protocol for the Fungal Mini Kit (Omega); subsequently, the DNA purity and total concentration were assessed via a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). ITS primers (ITS1 sequence: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 sequence: 5'- TCCTCCGCTTATTGATATGC-3') were used for DNA amplification (White *et al*. 1990). PCR conditions were carried out with a final volume of 25 µL as follows: 9 µL of ddH_2O, 1 µL of each primer, 1.5 µL of genomic DNA and 12.5 µL of master mix. The PCR procedure comprised several steps: 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. The effectiveness of the PCR for the amplification products and length were assessed by 1.5% agarose gel electrophoresis.

Sequencing results and phylogenetic analysis

Each PCR product was subjected to sequencing via Macrogen Company/South Korea according to the http://dna.macrogen.com requirements for the preparation and handling of

samples. The gene sequence was deposited in GenBank (https://www.ncbi.nlm.nih.gov/) under the specific accession number, followed by analyses using the BLAST search tool at NCBI. Highly similar hit sequences were downloaded from the GenBank database and aligned via Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic trees were constructed using MEGA 11 with the maximum likelihood method (Kumar *et al*. 2016).

Pathogenicity test

The pathogenicity test was performed in a greenhouse at 25 ± 2°C and 85% relative humidity. Seeds of the local variety of Faba bean were sterilized with 75% ethanol and used for pathogenicity experiments. The plants were subsequently seeded in sterilized vermiculite and soil pots with a diameter of 20 cm; subsequently, the plants were watered and monitored on a daily basis. The inoculum of each examined fungus was prepared according to (Razak and Abass 2021) by plating a fungal disc for pathogen growth (mycelium and conidia) on a PDA plate and cultured at 25 ± 1°C for 7 days. The mycelia and conidia were collected in ddH_2O and Tween 80 and adjusted to 1×10^6 cfu/mL using a hemocytometer. Ninety days after sowing, the Faba bean plants were inoculated with a fungal suspension at 1×10^6 cfu/mL, and dH_2O was used as a control treatment. The treated plants were covered with plastic bags for 48 hours to maintain a high humidity. After 14 days of infection, disease progression was evaluated by visualizing the disease symptoms and signs in the infected tissues and evaluating the conidia of the fungal pathogen via light microscopy, and the pathogen was reisolated from infected plant parts to apply Koch's postulates. The obtained results presented reflect the percentage of disease caused by each fungal species on the tested plants during the pathogenicity trial.

RESULTS AND DISCUSSION

Several disease symptoms on Faba bean aerial parts were observed during the period 2022-2023 in many Faba bean fields in Shaat-Al-Arab and Abu-Alkhaseeb in Basrah Province. The disease symptoms exhibited slight differences; generally, the initial symptoms started as small circular to irregular solitary spots with white colour in the centre and yellow to dark brown colour in the margins on the shoot system (leaves and stems) of Faba bean. Gradually, and under the favourable conditions of cold and humid weather, disease symptoms spread throughout the stems, and leaves developed progressively. The disease spots became black and increased in size with distinguished thicknesses and margins between the boundaries of infected and healthy tissues.

Under severe disease conditions, whole leaves are covered with necrotic white spots and exhibit symptoms of canker, which causes extensive leaf senescence and the death of infected plants (Figure 1). The current observations are in good agreement with those of many studies showing the virulence of several *Alternaria* species on economically important plants, including wheat, cumin, onion and tomato (Al-Nadabi *et al*. 2018; Abdel Aziz *et al*. 2021; Dominique *et al*. 2022; Htun *et al*. 2022).

Figure 1. Diseases symptoms of leaf and stem spot of Faba bean plants in fields**.**

Morphometric, microscopic and molecular identification of *Alternaria* isolates

***Alternaria alstroemeriae* E.G. Simmons & C.F. Hill**

Description: *A. alstroemeriae* colonies that grew on PDA plates were circular and reached their maximum growth (90 mm) after 7 days of incubation at 25 ± 1°C, the colour of colonies are olivaceous brown to black, the reverse growth colour was dark brown (Figure 2 A and B). The conidiophores were simple to branched, erect and straight; with the dimension of 10-33 X 3-5 µM, producing conidia in short chains of 2-5 (maximum 7), cylindrical to sub-cylindrical shape with 2-10 transverse septa and 0-2 longitudinal septa and smooth- walled. Conidia colours were yellowish brown to dark brown with apical beak (10-14 µM long and 2-3 µM thickness) and their sizes 15-72 X 6-16 µM (Figure 2 C). Morphometric and microscopic characteristics of *A. alstroemeriae* isolated form Faba bean spotted leaves were in consistent with previously description of (Yamagishi *et al*. 2009; Simmons 2007). The most important features of *A. alstroemeriae* are their uniqueness of short chain conidia with subcylidrical shape produced in basal parts of conidia chains.

Molecular identification: The PCR product of ITS gene sequence of Basrah isolate (*Alternaria alstroemeriae*) amplified a specific DNA fragment of 513 bp, the phylogenetic analysis reveals a

similarity percentage of 100% with the China isolate *A. alstroemeriae* (OP482338) which formed one subclade as depicted in Figure (2 D). The ITS gene sequence of Basrah isolate was deposited in GenBank under the accession number LC769965.1. The efficiency of ITS gene in confirmation of *A. alstroemeriae* has been proved in recent studies (Zhou *et al*. 2023).

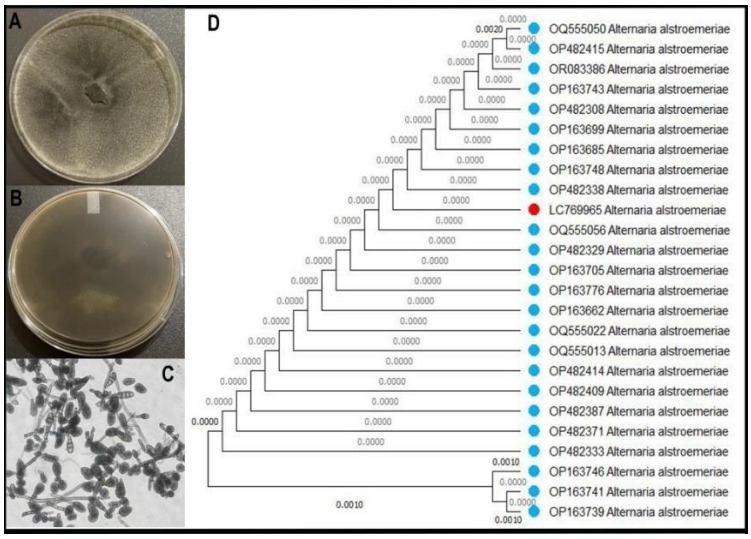

Figure 2. *Alternaria alstroemeriae* growth on PDA for 7 days at 25 °C; (A) Colony morphology (Top appearance), (B) Reverse growth, (C) Conidia and conidiophores. (D) Phylogenetic tree constructed by the neighbor–joining method using the ITS sequence of the *A. alstroemeriae* Basrah isolate (LC769965.1) with the nearest *A. alstroemeriae* published in GenBank: https://www.ncbi.nlm.nih.gov/genbank/samplerecord/.

Alternaria arborescens E.G. Simmons

Description: *A. arborescens* colonies that grew on PDA plates were circular and flat, reached their maximum growth (90 mm) after 6 days of incubation at 25 ± 1°C, the colour of colonies is greyish green to olivaceous brown, with white edges, the reverse side growth was yellowish brown to yellowish olivaceous colour (Figure 3 A and B). The hyphae were hyaline to light brown, septate. Conidiophores were solitary and straight, dark brown colour with the dimension of 50-200 µM; 2.5 µM thicknesses, producing conidia oval shape with 1-4 transverse septa and longitudinal septa. Conidia colour was dark brown with apical beak and their sizes 11-33 X 6-14 µM (Figure 3 C). Conidial chains were simple with 3-15 conidia. Morphometric and microscopic characteristics of *A. arborescens* isolated form Faba bean spotted leaves were in consistent with previously description of (Liao *et al*. 2023; Simmons 2007).

Molecular identification: The PCR amplification of ITS gene sequence of Basrah isolate (*Alternaria arborescens*) produced a specific amplicon of 513 bp, the phylogenetic analysis shows a similarity percentage of 100% with the China isolate *A. arborescens* (OR884103) which formed

one subclade as depicted in Figure (3 D). The ITS gene sequence of Basrah isolate was submitted in GenBank under the accession number LC769971.1. The current results are in a good agreement with the findings of (Liao *et al*. 2023) who revealed the identity of *A. arborescens* using ITS primers (ITS1 and ITS4).

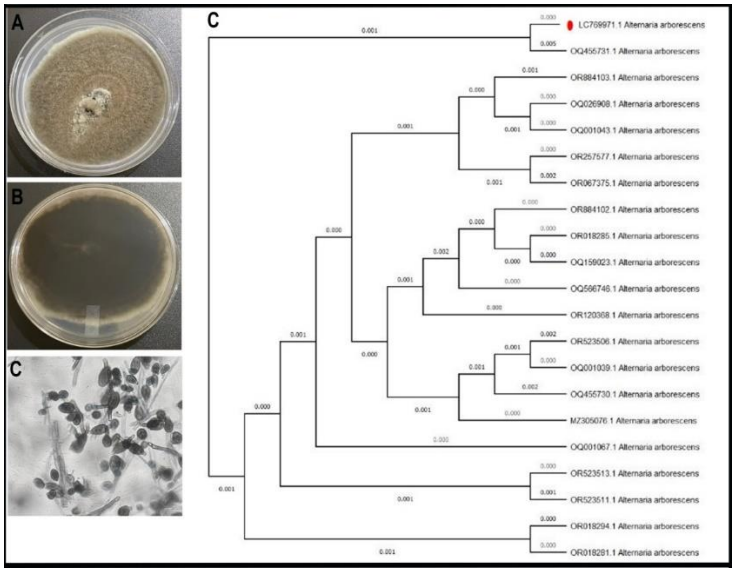

Figure 3. *Alternaria arborecsens* growth on PDA for 7 days at 25 °C**;** (A) Colony morphology (Top appearance), (B) Reverse growth, (C) Conidia and conidiophores. (D) Phylogenetic tree constructed by the neighbor–joining method using the ITS sequence of the *A. arborescens* Basrah isolate (LC769971.1) with the nearest *A. arborescens* published in GenBank: https://www.ncbi.nlm.nih.gov/genbank/samplerecord/.

Alternaria chlamydospora Mouch., 1973

Description: *A. chlamydospora* colonies that grew on PDA plates were circular, reached their maximum growth (90 mm) after 6-7 days of incubation at 25 ± 1°C, the colour of colonies is dark brown to olivaceous, the reverse side growth was black colour (Figure 4 A and B). Conidiophores were solitary and septate, simple to branched, smoothed to thick- walled dark brown colour with the dimension of 140 µM X 3-5 µM, producing conidia obpyriform shape, smooth- walled with 3-6 transverse septa and 2-3 longitudinal septa, constricted at the septa. Conidia colour was golden brown smooth- walled, with a pale beak (8 µM, 2-5 µM thickness), their sizes 18-55 X 7-35 µM (Figure 4 C). Chalmydopsores are produced with multi-cell and very variable shapes and sizes with golden colour (Figure 4 D). Morphometric and microscopic characteristics of *A. chlamydospora* isolated form Faba bean spotted leaves were in consistent with previously description of (Ellis 1971; Woudenberg *et al*. 2013).

Molecular identification: The PCR product of ITS gene sequence of Basrah isolate (*Alternaria chlamydospora*) amplified a specific DNA fragment of 543 bp, the phylogenetic analysis reveals a similarity percentage of 99% with the Iran isolate *A. chlamydospora* (KY788047) which formed one subclade as depicted in Figure (4 D). The ITS gene sequence of Basrah isolate was deposited in GenBank under the accession number LC769962.1. The efficiency of ITS gene in confirmation of *A. chlamydospora* identity has been shown in the study of (Attia *et al*. 2020).

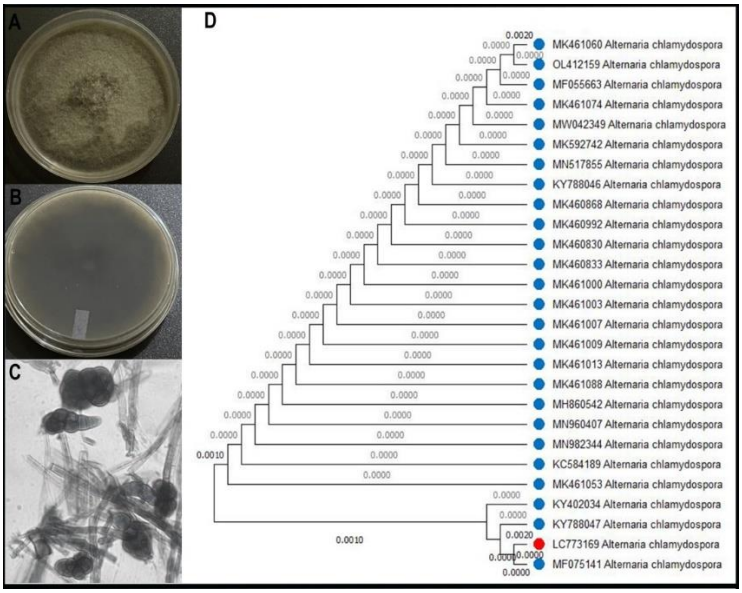

Figure 4. *Alternaria chlamydospora* growth on PDA for 7 days at 25 °C; (A) Colony morphology (Top appearance), (B) Reverse growth, (C) Conidia and conidiophores. (D) Phylogenetic tree constructed by the neighbor–joining method using the ITS sequence of the *A. chlamydospora* Basrah isolate (LC769962.1) with the nearest *A. chlamydospora* published in GenBank: https://www.ncbi.nlm.nih.gov/genbank/samplerecord/.

Alternaria concatenta Woudenb. & Crous

Description: *A. concatenta* colonies that grew on PDA plates were circular, reached their maximum growth (90 mm) after 7 days of incubation at 25 ± 1°C, the colour of colonies is dark brown to black, the reverse side growth was brown colour (Figure5 A and B). Conidiophores were solitary and straight, simple to branched, curved, smoothed to thick- walled dark brown colour with the dimension of 35-60 µM X 3-5 µM, producing single conidium oval shape with 2-3 transverse septa and 1-2 longitudinal septa. Conidia colour was dark brown smooth- walled and beakless, their sizes 12-30 X 8-18 µM (Figure 5 C). Morphometric and microscopic characteristics of *A. concatenta* isolated form Faba bean spotted leaves were in consistent with previously description of (Woudenberg *et al*. 2013).

Molecular identification: The PCR amplification of ITS gene sequence of Basrah isolate (*Alternaria concatenta*) produced a specific DNA fragment of 515 bp, the phylogenetic analysis proves a similarity percentage 99% with the Iran isolate *A. concatenta* (KY788030) which formed one subclade as depicted in Figure (5 D). The ITS gene sequence of Basrah isolate was deposited in GenBank under the accession number LC769963.1. The current results are in a good agreement with the findings of (Liao *et al*. 2023) who revealed the efficiency of ITS primers (ITS1 and ITS4) in fungal identification.

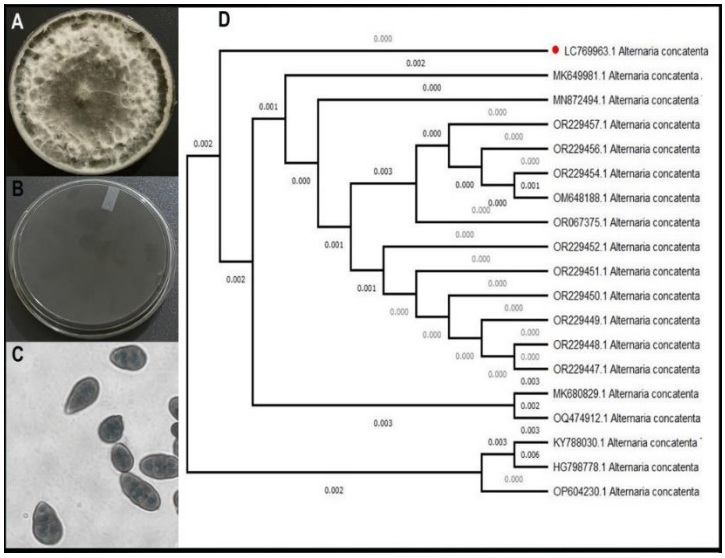

Figure 5. *Alternaria concatenta* growth on PDA for 7 days at 25 °C; (A) Colony morphology (Top appearance), (B) Reverse growth, (C) Conidia and conidiophores. (D) Phylogenetic tree constructed by the neighbor–joining method using the ITS sequence of the *A. concatenta* Basrah isolate (LC769963.1) with the nearest *A. concatenta* published in GenBank: https://www.ncbi.nlm.nih.gov/genbank/samplerecord/.

Alternaria gaisen Nagano ex Hara, Sakumotsu Byorigaku, Edn.

Description: *A. gaisen* colonies that grew on PDA plates produces distinct concentric rings representing zones of abundant conidiophore and conidia, reached their maximum growth (90 mm) after 7 days of incubation at 25 ± 1° C, the colour of colonies is dark greenish to black, the reverse side growth was black colour (Figure 6 A and B). Conidiophores were solitary, septate, simple to branched, straight or curved, smoothed to thick- walled pale olivaceous colour with the dimension of 30-40 µM X 5-2 µM, producing conidia in chains of 4-10, ovoid to ellipsoid shape, smooth-walled with 3-5 transverse septa and 1-2 longitudinal septa. Conidia colour was yellowish brown, with very short pale beak (4 µM thickness) or beakless, their sizes 18-50 X 8-17 µM (Figure 6 C).

Morphometric and microscopic characteristics of *A. gaisen* isolated form Faba bean spotted leaves were in consistent with previously description of (Woudenberg *et al*. 2013; Akhtar *et al*. 2014).

Molecular identification: The PCR amplification of Basrah isolate (*Alternaria gaisen*) using ITS primers (ITS1 and ITS4) amplified a specific DNA product in the size of 516 bp, the phylogenetic analysis reveals a similarity percentage of 99% with the China isolate *A. gaisen* (AF314581) which formed one subclade as depicted in Figure (6 D). The ITS gene sequence of Basrah isolate was deposited in GenBank under the accession number LC769957.1. Molecular identification using gene sequencing to identify *A. gaisen* as a pathogen on plant has been cited in many recent studies such as the study of (Kawashimo and Sakurai 2024).

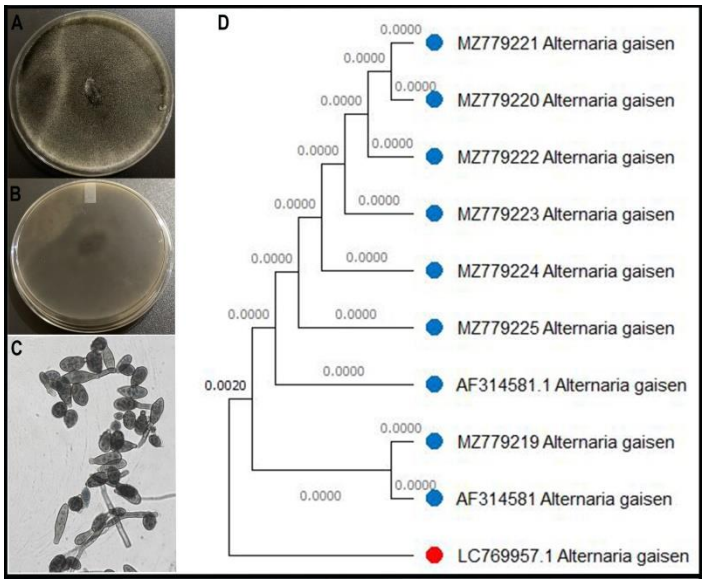

Figure 6. *Alternaria gaisen* growth on PDA for 7 days at 25 °C; (A) Colony morphology (Top appearance), (B) Reverse growth, (C) Conidia and conidiophores, (D) Phylogenetic tree constructed by the neighbor–joining method using the ITS sequence of the *A. gaisen* Basrah isolate (LC769957.1) with the nearest *A. gaisen* published in GenBank: https://www.ncbi.nlm.nih.gov/genbank/samplerecord/.

Alternaria infectoria Simmons

Description: *A. infectoria* colonies that grew on PDA plates reached their maximum growth (90 mm) after 7 days of incubation at 25 ± 1°C, the colour of colonies is greyish and later turned black, the reverse side growth was black colour (Figure 7 A and B). Conidiophores were solitary, septate, simple to branched, straight or curved, smoothed to thick- walled greyish colour with the dimension of 60-80 µM X 3-5 µM, producing conidia in chains of 4-10, ovoid to ellipsoid shape, thick- walled with 4-7 transverse septa and 2-4 longitudinal septa. Conidia colour was brown to

dark brown, with short apical beak (4 µM thickness), their sizes 18-60 X 8-16 µM (Figure 7 C). Morphometric and microscopic characteristics of *A. infectoria* isolated form Faba bean spotted leaves were in consistent with previously description of (Andersen and Thrane 1996; Simmons 2007).

Molecular identification: The PCR product of ITS gene sequence of Basrah isolate (*Alternaria infectoria*) amplified a specific DNA fragment of 502 bp, the phylogenetic analysis reveals a similarity percentage of 100% with the Germany isolate *A. infectoria* (MT561399) which formed one subclade as depicted in Figure (7 D). The ITS gene sequence of Basrah isolate was deposited in GenBank under the accession number LC769961.1. The ITS gene efficiency in *A. infectoria* identification is in accordance with the results of (Moslemi *et al*. 2017) in their study on pyrethrum (*Tanacetum cinerariifolium*) flowers.

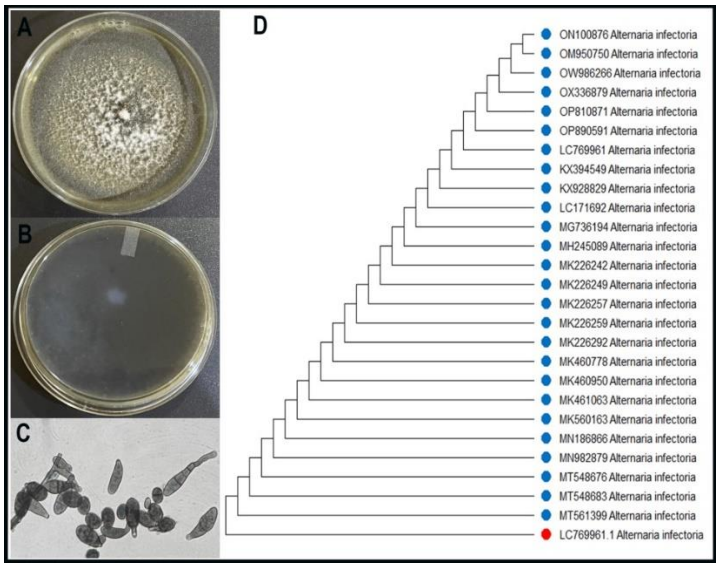

Figure 7. *Alternaria infectoria* growth on PDA for 7 days at 25 °C; (A) Colony morphology (Top appearance), (B) Reverse growth, (C) Conidia and conidiophores, (D) Phylogenetic tree constructed by the neighbor–joining method using the ITS sequence of the *A. infectoria* Basrah isolate (LC769961.1) with the nearest *A. infectoria* published in GenBank: https://www.ncbi.nlm.nih.gov/genbank/samplerecord/.

Alternaria porri Simmons

Description: *A. porri* colonies that grew on PDA plates reached their maximum growth (90 mm) after 7 days of incubation at 25 ± 1° C, the colour of colonies is greyish and later turned black, the reverse side growth was black colour (Figure 8 A and B). Conidiophores were solitary, septate, simple to branched, straight or curved, smoothed- walled greyish colour with the dimension of 90-110 µM X 5-8 µM, producing single conidium, straight to curved shape, smooth- walled with 7-

11 transverse septa and 1-2 longitudinal septa. Conidia colour was pale brown to dark brown, with long curved beak (5 µM thickness) with transverse septa, their sizes 70-250 X 14-22 µM (Figure 8 C). Morphometric and microscopic characteristics of *A. porri* isolated form Faba bean spotted leaves were in consistent with previously description of (Mohsin *et al*. 2016; Simmons 2007).

Molecular identification: The PCR amplification of Basrah isolate (*Alternaria porri*) using ITS primers (ITS1 and ITS4) amplified a specific DNA product in the size of 507 bp, the phylogenetic analysis reveals a similarity percentage of 100% with the India isolate *A. porri* (MK224472) which formed one subclade as depicted in Figure (8 D). The ITS gene sequence of Basrah isolate was deposited in GenBank under the accession number LC769959.1. The current results are in agreement with the study by (Gou *et al*. 2023) of the efficiency of ITS gene in *Alternaria* species identification

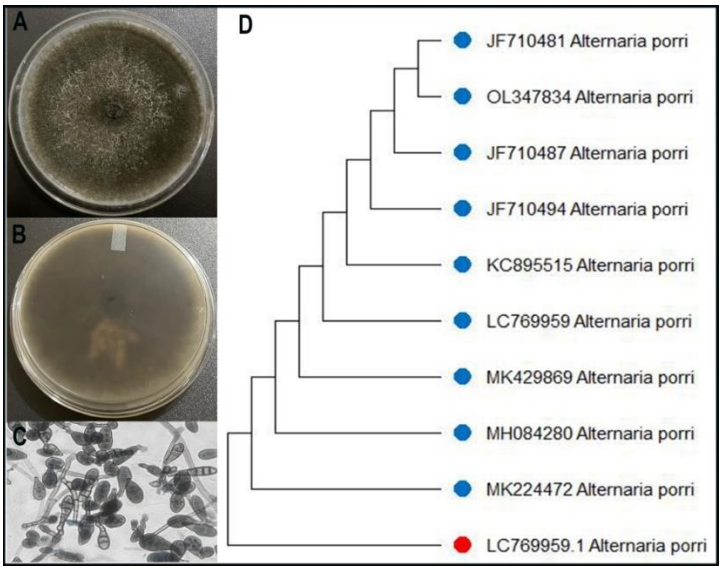

Figure 8. *Alternaria porri* growth on PDA for 7 days at 25 °C; (A) Colony morphology (Top appearance), (B) Reverse growth, (C) Conidia and conidiophores, (D) Phylogenetic tree constructed by the neighbor–joining method using the ITS sequence of the *A. porri* Basrah isolate (LC769959.1) with the nearest *A. porri* published in GenBank: https://www.ncbi.nlm.nih.gov/genbank/samplerecord/.

Alternaria terricola Woudenb. & Crous

Description: *A. terricola* colonies that grew on PDA plates reached their maximum growth (90 mm) after 7 days of incubation at 25 ± 1°C, the colour of colonies is black, the reverse side growth was black colour (Figure 9 A and B). Conidiophores were solitary, septate, simple to branched, mostly curved, smoothed- walled brown colour with the dimension of 30-50 µM X 2-4 µM, producing conidia single or in short chains, smooth- walled, obovoid with 1-3 transverse septa and

1-2 longitudinal septa. Conidia colour was pale brown to dark brown, beakless, their sizes 12-20 X 8-12 µM (Figure 9 C). Morphometric and microscopic characteristics of *A. terricola* isolated form Faba bean spotted leaves were in consistent with previously description of (Woudenberg *et al*. 2013; Cherif *et al*. 2022).

Molecular identification: The PCR amplification of ITS gene sequence of Basrah isolate (*A. terricola*) produced a specific DNA amplicon of 483 bp, the phylogenetic analysis reveals a similarity percentage of 99% with the India isolate *A. terricola* (KY788080) which formed one subclade as depicted in Figure (9 D). The ITS gene sequence of Basrah isolate was deposited in GenBank under the accession number LC769960.1. The results of ITS gene efficiency in *A. terricola* identification is in accordance with the results of (Cherif *et al*. 2022). The results of *Alternaria* species identities and similarities are shown in Table (2 and 3).

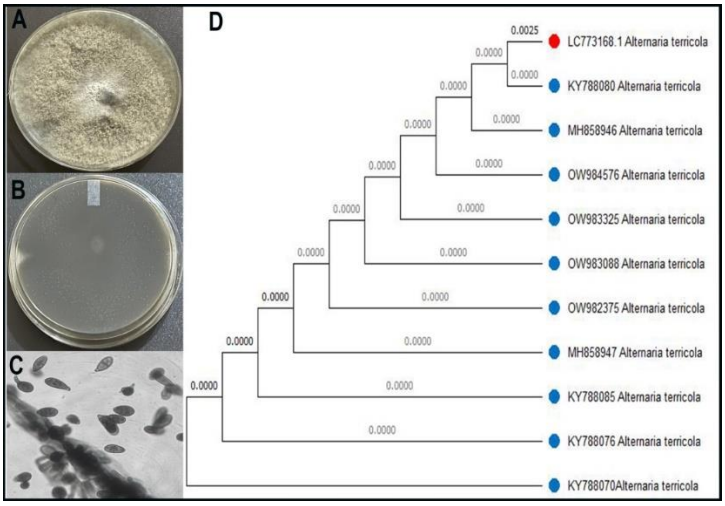

Figure 9. *Alternaria terricola* growth on PDA for 7 days at 25 °C**;** (A) Colony morphology (Top appearance), (B) Reverse growth (C) Conidia and conidiophores, (D) Phylogenetic tree constructed by the neighbor–joining method using the ITS sequence of the *A. terricola* Basrah isolate (LC769960.1) with the nearest *A. terricola* published in GenBank: https://www.ncbi.nlm.nih.gov/genbank/samplerecord/.

Table 1. Morphological comparison of *Alternaria* species isolated from Faba bean aerial parts.

***Alternaria* species**	**Conidia description**				
	Shape	**Body/ µM**	**Septa**		Beak/ µM
			Transverse	**Longitudinal**	
A. alstroemeriae	Cylindrical to sub-cylindrical	15-72 X 6-16	2-10	0-2	Long (10-14)
A. arborescens	Oval	11-33 X 6-14	1-4	1-4	Apical beak
A. chlamydospora	Obpyriform	18-55 X 7-35	3-6	2-6	Pale (8)
A. concatenta	Oval	35-60 X 3-5	2-3	1-2	Beakless
A. gaisen	Ovoid to ellipsoid	18-50 X 8-17	3-5	1-2	Beakless
A. infectoria	Ovoid to ellipsoid	18-60 X 8-16	4-7	2-4	Short (4)
A. porri	Curved	70-250 X 14-22	7-11	1-2	Long (5)
A. terricola	Obovoid	12-20 X 8-12	1-3	1-2	Beakless

Table 2. Molecular identification results of *Alternaria* species isolated from Faba bean aerial parts.

Alternaria species of presented study	Gene Accession Number	ITS Sequence Size/ bp	Nearest Gene Accession Number	%Similarity	Country
A. alstroemeriae	LC769965.1	513	OP482338	100	China
A. arborescens	LC769971.1	513	OR884103	100	China
A. chlamydospora	LC769962.1	543	KY788047	99	Iran
A. concatenta	LC769963.1	515	KY788030	99	Iran
A. gaisen	LC769957.1	516	AF314581	99	China
A. infectoria	LC769961.1	502	MT561399	100	Germany
A. porri	LC769959.1	507	MK224472	100	India
A. terricola	LC769960.1	483	KY788080	99	Iran

Pathogenicity test

Pathogenicity trial results under greenhouse conditions using a local sensitive cultivar of Faba bean showed that all examined isolates of *Alternaria* species (*A. alstroemeriae*, *A. arborescens*, *A. chlamydospora*, *A. concatenta*, *A. gaisen*, *A. infectoria*, *A. porri* and *A. terricola*) were able to induce disease symptoms (Figure 10 A-H). Earlier symptoms appeared on plant aerial parts 14 days postinoculation as oval to round white spots with different lesion sizes ranging from 0.1 to 1 cm on leaf surfaces. These spots soon merge to form large and change to brown or black similar to what was reported in the Faba bean fields in Shaat-Al-Arab and Abu-Alkhaseeb in Basrah Province. On the other hand, the untreated control plants (inoculated with dH_2O) remained healthy during the pathogenicity trial. The results were consistent with those of the *Alternaria* species isolates when reisolated from infected tissues of Faba bean plants, which confirmed Koch's postulates.

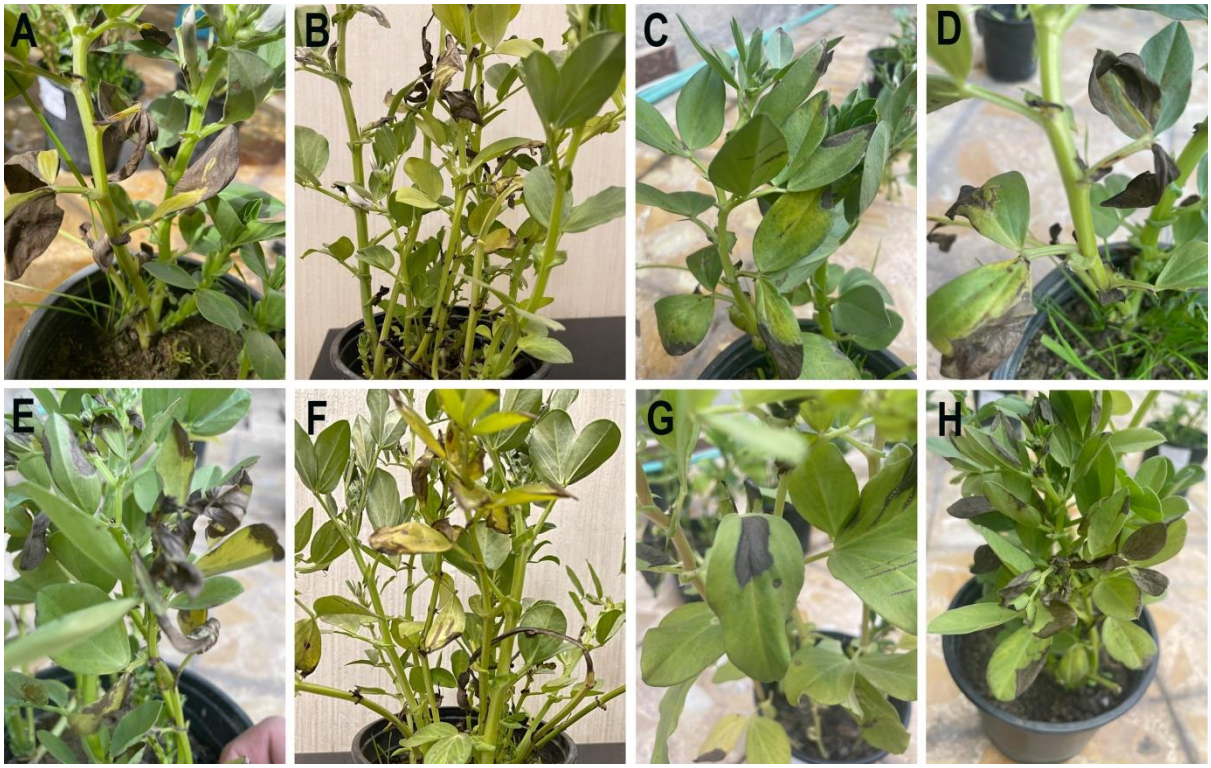

Figure 10. Pathogenicity trial is using *Alternaria* species on a sensitive cultivar of Faba bean; (A) *A. alstroemeriae*, (B) *A. arborescens*, (C) *A. chlamydospora*, (D) *A. concatenta*, (E) *A. gaisen*, (F) *A. infectoria*, (G) *A. porri*, and (H) *A. terricola*.

The results of the pathogenicity test were in good agreement with many studies reporting the pathogenicity of all examined *Alternaria* species on economic plants (Coca-Morante and Mamani 2012; Al-Nadabi *et al*. 2018; Abdel Aziz *et al*. 2021; Bankina *et al*. 2021; Razak and Abass 2021; Dominique *et al*. 2022; Htun *et al*. 2022; Yaser and Abass 2022). This study is the first to report *Alternaria* species (*A. alstroemeriae, A. arborescens, A. chlamydospora, A. concatenta, A. gaisen, A. infectoria, A. porri* and *A. terricola*) as potential pathogens on Faba bean aerial parts. The pathogenicity effects of *Alternaria* species on Faba bean tissues could be explained by their ability to produce several important toxins and hydrolytic enzymes, in addition to their ability to be transmitted in seeds as a seed-borne pathogen (Sharma and Pandey 2013; Khare *et al*. 2014; Singh *et al*. 2016).

CONCLUSIONS

The objective of the present study was to isolate and identify the *Alternaria* spp. fungal pathogen that causes Faba bean aerial parts spot disease in Basrah Province, Iraq. To the best of our knowledge, this study is the first to report *A. alstroemeriae, A. arborescens, A. chlamydospora*,

A. concatenta, A. gaisen, A. infectoria, A. porri and *A. terricola* as true pathogens of Faba bean spot disease in Iraq. Molecular identification has been applied to reveal the identity of *Alternaria* spp. via the ITS gene sequence, and all of the examined ITS sequences have been deposited in NCBI-BLAST under accession numbers LC769965.1, LC769971.1, LC769962.1, LC769963.1, LC769957.1, LC769961.1, LC769959.1 and LC769960.1 for *Alternaria* species. Further studies are needed to determine the best control measures to restrict the development of these disease pathogens on Faba bean plants.

Author Contributions

Conceptualization, (B. G. O.) carried out the experiments, (Y. A. S.) identified the fungal species; (M. H. A.) took the lead in writing of manuscript (Y. A. S.) and (M. H. A.) contributed to the reviewing and editing of the manuscript. All the authors discussed the experiment results and agreed to the published version of the manuscript.

Data Availability Statement

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Consent to participate

Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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