Morphological and Molecular Identification, Pathogenicity Analyses and Prevalence of *Alternaria alternata* in Wheat Fields of Karbala Province, Iraq

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Abstract

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Alternaria alternata is well-known as a causal agent of various plant diseases worldwide. However, the species distribution causing the failure of wheat seed germination in Iraq has not been studied. During a field survey, one hundred seventy-two *Alternaria* isolates were collected from 69 wheat fields in Karbala province, Iraq in 2022-2023. Based on morphological features and sequence analysis of different genetic markers included the internal transcribed spacer (ITS), the DNA-directed RNA polymerase I (RPB1) and DNA-directed RNA polymerase II (RPB 2), the translation elongation factor (TEF) gene, and the beta-tubulin gene (β-tubulin) were determined. The majority of the *Alternaria* isolates obtained were recognized as *A. alternata*. To the best of our knowledge, this is the first description of *A. alternata* as the leading cause of seed decay and damping-off of wheat crop in Karbala province of Iraq.

Keywords: *Alternaria alternata*, morphology, wheat, pathogenicity, phylogeny

Introduction

Wheat, *Triticum aestivum* L. is one of the most economically important cereal crops worldwide, belonging to the genus *Triticum* within the Poaceae family (Haas *et al.,* 2019). Globally, wheat is the second most widely cultivated cereal after maize, with major producing countries including China, India, Russia, the United States, and France (FAO, 2023). In Iraq, wheat is a vital agricultural crop, forming a cornerstone of food security and staple food production (Lahuf *et al.,* 2018a). In 2020, the country had a cultivated area of approximately 2.1 million hectares dedicated to wheat production, primarily for domestic consumption as bread and other traditional food. The annual production of wheat in Iraq is around 6.2 million tonnes, making it one of the most significant cereal crops in the region and essential for meeting the nation's dietary and economic needs (Ministry of Agriculture of Iraq, 2020).

Wheat crops are susceptible to a significant number of biotic and abiotic stresses, with fungal diseases being the most important. These stresses can severely impact yield and quality, particularly under unfavourable environmental conditions (Chen, 2005). Major fungal and fungal-like diseases affecting wheat production globally, including Iraq, are Fusarium head blight (*Fusarium graminearum* Schwabe), leaf rust (*Puccinia recondita* Roberge ex Desm.), stripe rust (*P. striiformis* f. sp. *tritici*), stem rust (*P. graminis* f. sp. *tritici*), powdery mildew (*Blumeria graminis* f. sp. *tritici*), and take-all (*Gaeumannomyces graminis* var. *tritici*). These pathogens cause substantial yield losses and threaten food security, especially in areas with limited resources to implement effective management strategies (Bockus *et al.,* 2015; Jaber & Lahuf, 2020; Singh *et al.,* 2011).

Alternaria species are universal fungal pathogens and saprotrophs distributed worldwide in various environments. They can infect numerous vegetables, oil, cereal and medicinal plants, including wheat crops (Alamry *et al.,* 2024; Gao *et al.,* 2021; Hameed *et al.,* 2021; Lahuf *et al.,* 2018b). One harmful species is *A. alternata,* whose interaction with wheat significantly affects production, primarily due to its role in seed decay and damping-off diseases (Mercado *et al.,* 2006). *A. alternata* is recognized as a widespread fungal pathogen capable of infecting a range of plant species (Lahuf *et al.,* 2020), including wheat, resulting in significant economic yield loss. This pathogen is mainly responsible for black point disease in wheat, which can significantly reduce seed quality and germination rates, impacting overall crop yields (Adhikari *et al.,* 2016; Bensassi *et al.,* 2009; Hussain *et al.,* 2013; Turzhanova *et al.,* 2020). The existence of this fungus in wheat seeds has been related to substantial yield losses, ranging from 15 to 90%, mainly with untreated wheat seeds (Adhikari *et al.,* 2018; Hussain *et al.,* 2013). Additionally, *A. alternata* fungus can produce mycotoxins, such as alternariol and alternariol monomethyl ether, which can cause additional harmful risks to both human and livestock health (Bagi *et al.,* 2022; Meena *et al.,* 2017).

The relationship between *A. alternata*, seed decay, and damping-off in wheat highlights the need to investigate the distribution and pathogenicity of this pathogen on wheat fields in Karbala province, which has been recently considered one of the main wheat producing regions in Iraq. Thus, this study aimed to isolate and identify the *A. alternata* pathogen associated with seed decay and damping-off wheat crops, based on morphological and phylogenetic analysis, and to confirm their pathogenicity and virulence on wheat cultivars that are common in Karbala Province, Iraq.

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Materials and Methods

Collection of samples, isolation, and purification

Wheat seed samples harvested in the agricultural season 2022-2023 (500g/field) were collected randomly from 69 fields spread across the districts and sub-districts of Karbala province. Samples were packed in tightly sealed plastic bags with all related information marked on them. These samples were then transferred to the laboratory and stored in the refrigerator at 4°C. The seed samples were washed thoroughly using tap water, then superficially sterilized with 2% sodium hypochlorite solution for three minutes, and then washed well with distilled water, sterilized with 70% ethanol for half a minute, and dried using filter paper (Lahuf, 2019a; Mehdi *et al.,* 2018). Subsequently, the sterilized seeds were transferred using sterile forceps to plastic Petri dishes with 8.5 cm in diameter containing prepared water agar medium (WA), were prepared with five seeds in each Petri dish. Dishes were then incubated at 25±2°C for 3 days (Jasim *et al.,* 2019). After observing the fungal growth around the seeds, a disk (5 mm) was taken from the edge of each fungal colony and planted in Petri dishes containing potato dextrose agar medium (PDA) for purification, then incubated at the same temperature mentioned above (Burns, 2009; Lahuf *et al.,* 2022; Watanabe, 2010).

Morphological identification

The initial assessment of fungi resembling *Alternaria* spp. was carried out as described by Basim *et al.* (2017). Among 172 fungal isolates obtained, the suspected Alternaria cultures were sustained on PDA at 25°C. The morphology of the emerging colonies was examined. The size and morphological characteristics were then analyzed, including colony colour, margin, and texture (Deng *et al.,* 2013). The sporulation patterns and the characteristics of conidia (n = 50) were analyzed using light microscopy (Nikon, Tokyo, Japan) and documented through photography (Yassin & Mohammed, 2021). These morphological features were compared with previous descriptions by Watanabe (2010) and Simmons (2007).

Molecular identification

Polymerase chain reaction (PCR) test and determination of the five selected genetic markers' nucleotide sequences were used to confirm the morphological identification of *Alternaria* fungal isolates associated with wheat seeds as described by Narayanasamy (2011). The PCR test used two microliters of genomic DNA extracted from each fungal isolate isolated from wheat seeds, and applied on 50 different isolates. The Ready-To-Go PCR Beads kit was used, which included the essential components for obtaining the PCR products, in addition to the genetic marker primers used in molecular identification, which amplify different genetic regions of the fungal genome (Lahuf *et al.,* 2019).

The internal transcribed spacer (ITS) region of rDNA was amplified via ITS1 and ITS4 primers (White *et al.* 1990); the DNA-directed RNA polymerase I (RPB1) and DNA-directed RNA polymerase II (RPB 2) were amplified via RPB1-Af and RPB1-Cr and RPB1-5F and RPB1-7R respectively (Matheny, 2005). The translation elongation factor (TEF) gene was amplified via EF1-728 F and EF1-986 R primers (Carbone & Kohn, 1999); and the beta-tubulin gene (β-tubulin) was amplified via BT2F and BT2R primers (Glass & Donaldson, 1995).

PCR amplicons were sent to Macrogen, South Korea, for sequencing. The sequencing data received were evaluated, cleaned and analyzed using the ApE plasmid Editor program. Then, the Basic Local Alignment Search Tool (BLAST) program was employed to determine the similarity between the sequences of the studied Alternaria fungi and the globally identified fungi stored in the GenBank repository of the National Center for Biotechnology Information (NCBI). The results of this analysis were also used to build a phylogenetic tree that showed the extent of convergence between local and global fungal isolates using the Molecular Evolutionary Genetics Analysis (MEGA) program version 10. The sequences of the nucleotide of the local fungal isolates were then deposited in the GenBank repository after assigning accession numbers to them (Lahuf, 2019b).

Pathogenicity assessment

The plate method was applied to determine the pathogenicity of Alternaria fungi associated with wheat seeds (Christensen *et al.,* 1988). This was done by taking a disc (5 mm in diameter) from the edge of pure 7 days old fungal colonies grown on PDA medium and placed in the middle of a plastic Petri dish containing the prepared WA medium. All inoculated dishes were incubated for 3 days at 25±2°C. The cultivar Iba 99 wheat seeds were then planted on the edges of the fungal colonies grown in the dish (10 seeds/dish). Each fungal isolate was repeated three times, in addition to the control treatments by planting sterile wheat seeds of the same variety without fungal colonies. All plates were incubated at 25±2°C until all wheat seeds in the control treatment germinated. The germination rate was then calculated as follows (Jaber & Lahuf, 2020):

$$\text{Germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

Another pathogenicity assessment was conducted using plastic pots with dimensions of 8×8×10 cm filled with mixed soil and peat moss fertilizer (1:1) and sterilized with 70% ethanol. Wheat seeds of three varieties were used, varying in their sensitivity, including Rashid, Ibaa 99 and Wafiya. The treatments were then carried out, with each treatment repeated three times. The sterilized wheat seeds were inoculated with the previously prepared pathogenic fungal inoculum at a concentration of 1×10^6 conidia/ml by soaking them for 2 hours, then planted at a rate of 10 seeds per pot. However, the control pots were planted with sterilized seeds without inoculum (Jaber, 2020).

Two weeks after planting date, the germination rate was calculated using the above equation. Four weeks after planting, the disease incidence (%) was calculated according to the following equation:

$$\text{Disease incidence (\%)} = \frac{\text{number of infected seedlings}}{\text{total number of seedlings}} \times 100$$

Results

Morphological and molecular identification

Seed decay and damping-off symptoms were observed in numerous surveyed wheat fields. Out of 172 fungal isolates obtained from diseased wheat seeds, 120 were identified as members of the *Alternaria* genus. This initial morphological identification depended on cultural and microscopic characterizations (Simmons, 2007). After one week, all of these Alternaria isolates developed aerial mycelium colonies in dark grey to olive brown color, with white edges. The mycelium growth converted to dark brown after two weeks. The average radial growth of the colony was 4.9 cm. The conidia, born on light brown conidiophores, were ellipsoidal or ovoid with 1–4 transverse septa, 24-28 µm long and 9-12 µm wide.

The sequences of ITS marker genes of the 120 isolates of *Alternaria* spp. obtained in the current study were subjected to a BLAST analysis with NCBI-GenBank. The similarity of these isolates was very high (98-100%) compared with standard Alternaria species. The majority of them (45 out of 120 isolates) belonged to *Alternaria alternata*, followed by *A. chlamydosporigena* and *A. sorghi* (16 out of 120 isolates for each species), then *A. cumini* (12 out of 120 isolates), in addition to other Alternaria species (Table 1).

To confirm the identification of *A. alternata* isolates, the cultural and microscopic features of the 45 isolates were further examined. The pure colony of these species was cottony, olive brown colour and had slight concentric growth circles (Figure 1-A). These isolates generated a conidial series of 5–10 conidia with many derived branching carried on little conidiophores. Conidia were obpyriform to ellipsoid with 1–4 transverse septa and 0-3 longitudinal septa. The average size was 26 µm long and 10 µm wide (Figure 1-B).

A multilocus identity and phylogenetic analyses were conducted utilizing five genetic markers (ITS, RPB1, RPB2, TEF, beta-tubulin). These analyses showed that the isolated *A. alternata* was similar to several global isolates of the same species by showing 100% similarity in ITS and TEF marker sequences and 99% similarity in RPB 1, RPB2 and β-tubulin sequences. Likewise, the phylogenetic tree among these marker sequences and other related sequences revealed the close relationship between the isolated *A. alternata* and many universal isolates and strains belonging to the same species by clustering them in the same clade. However, the other out-of-group fungi were placed in another clade.

Based on these characteristics and analyses that agreed with what has been reported by description of Nishikawa & Nakashima (2019), the Iraqi fungal isolate was confirmed to be *A. alternata* (Fries) Kessler. Thus, the representative sequence of each of the above marker genes was deposited to the NCBI-GenBank database with the name Karbala-1 and several accession numbers were assigned (ITS-PQ217832, RPB1-PQ212707, RPB2- PQ212708, TEF-PQ212710, β-tubulin- PQ212709) (Figures 2).

Table 1. *Alternaria* species isolated in this study.

Species	Number of isolates
Alternaria alternata	45
Alternaria chlamydosporigena	16
Alternaria sorghi	16
Alternaria cumini	12
Alternaria japonica	10
Alternaria tenuissima	10
Alternaria hungarica	7
Alternaria lolii	3
Alternaria rosae	1
Total	**120**

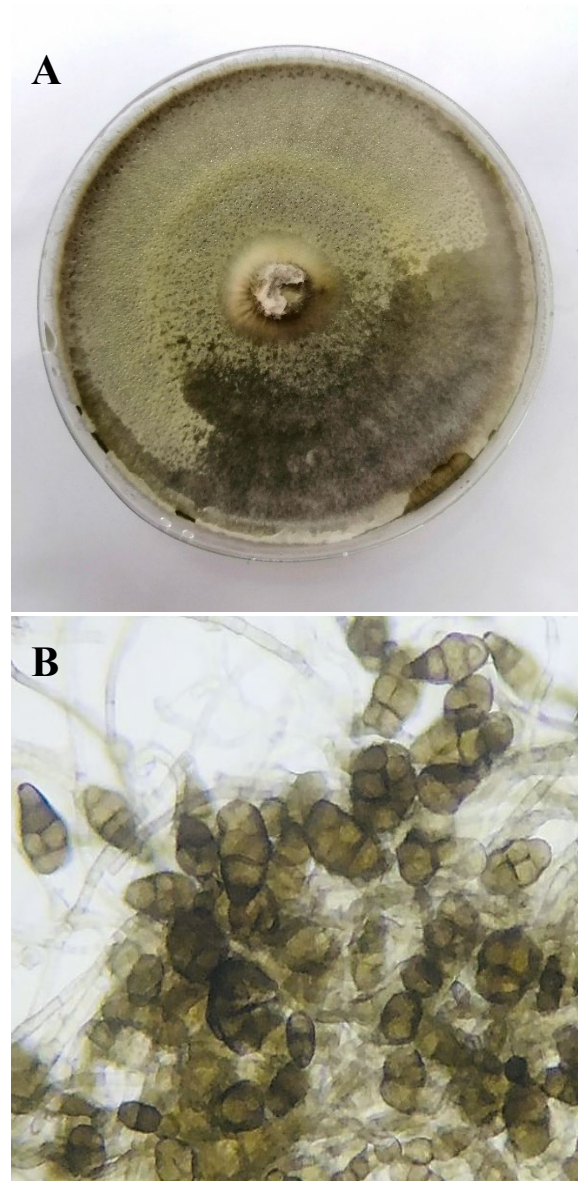


Figure 1. Cultural and microscopic features of *A. alternata* isolate Karbala-1. (A) colony growth of *A. alternata* isolate Karbala-1 after 7 days on PDA, (B) conidia of *A. alternata* isolate Karbala-1 found after two weeks on PDA. Scale bar = 20 µm.

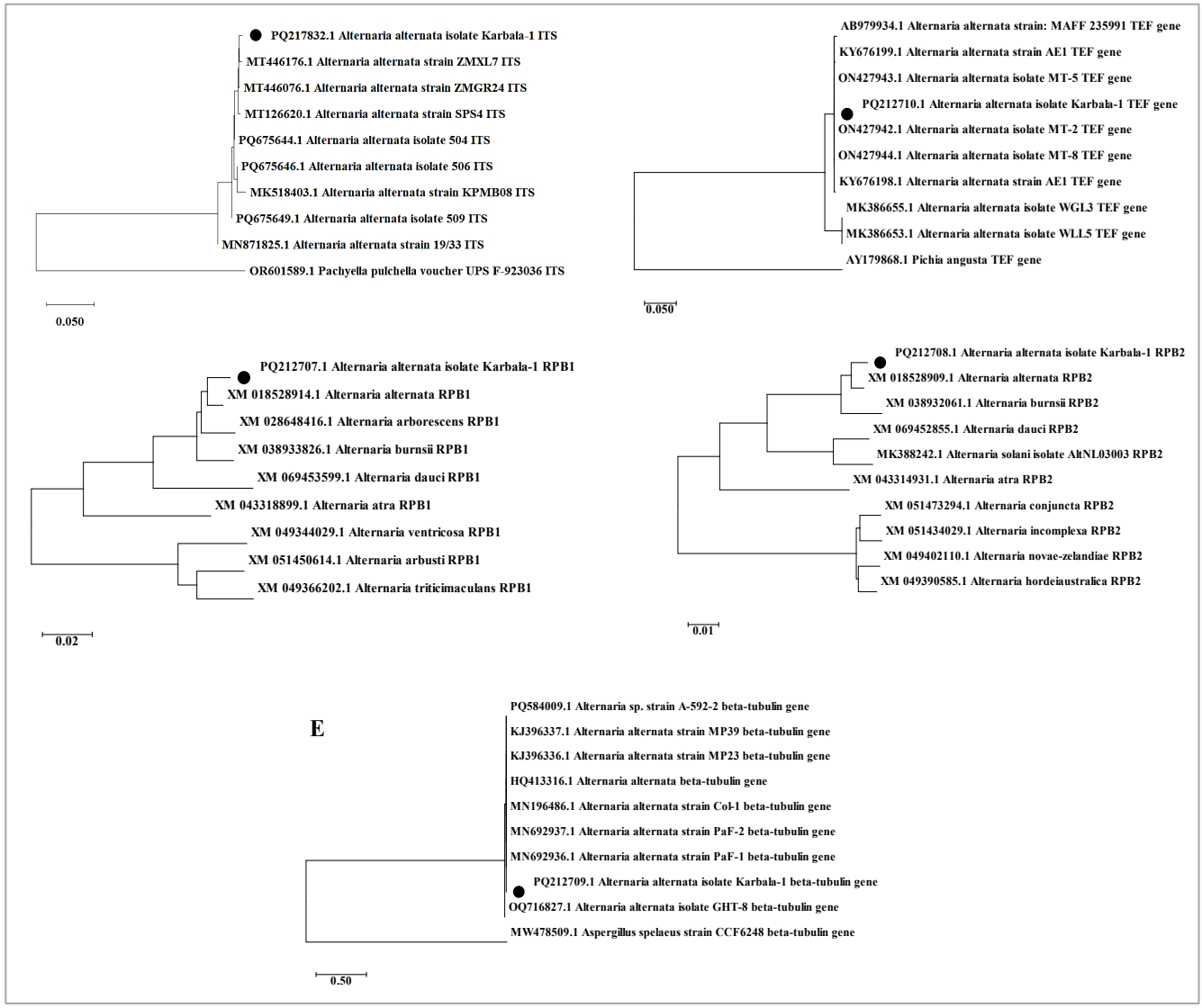


Figure 2. Phylogenetic tree illustrating the evolutionary relationships among *Alternaria alternata* isolate Karbala-1 (highlighted with a black dot) and other related isolates and strains based on (A) ITS marker sequences similarity; (B) TEF marker sequences similarity, (C) RPB1 marker sequences similarity, (D) RPB2 marker sequences similarity, (E) beta-tubulin marker sequences similarity.

Pathogenicity assessment

The examined Alternaria isolates were pathogenic on artificially inoculated wheat seeds by causing 100% infection. Symptoms appeared on wheat seeds infected by *Alternaria* spp., including *A. alternata,* which have various symptoms, including dark brown to black discolouration at the germ tube end and black, brown, or dark grey patches on the seed's coat. Infected seeds have a small, shell-like appearance, shriveled, and have a deformed shape. The seed surface was often fluffy or powdery in texture. In the most severe instances, seeds were soft and may ultimately lose their tissue. These symptoms can lead to lack of germination or produce seedlings that have malformatted roots and shoots. The seed's surface appeared rough, scabby, or chalky, suggesting the fungus presence (Figure 3). To achieve Koch's requirements, re-isolation was performed on all inoculated symptomatic seeds, and the isolated fungi were identified as being *Alternaria* spp. germinated healthy control seeds were symptomless.

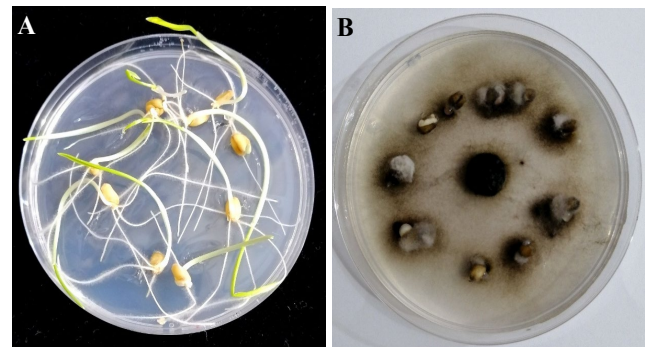


Figure 3. Pathogenicity of the representative isolates of *A. alternata* on wheat seeds. (A) Germinated healthy control seeds, (B) Disease symptoms of *A. alternata* on wheat seeds.

On the other hand, the pathogenicity assessment of *A. altenata* isolate Karbala-1 conducted in plastic pots showed the pathogenic effect on the seeds of three local varieties (Rashid, Ibaa 99 and Wafiya). The highest disease incidence (Figures 4 and 5) was noticed in Rashid variety by reaching the highest inhibition rate of 40%, followed by Ibaa 99 and Wafiya varieties at 33% and 10%, respectively.

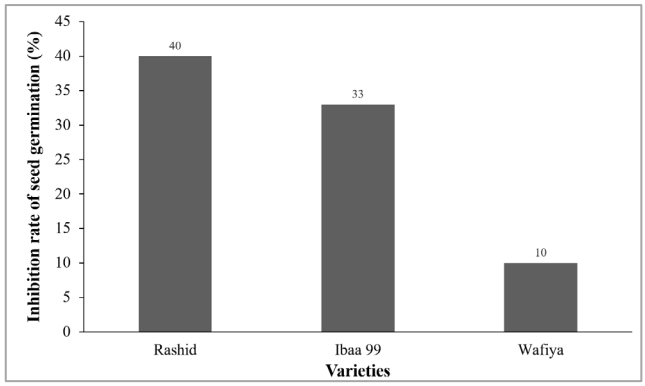


Figure 4. The inhibition rate of seed germination (%) caused by *A. alternata* on three Iraqi local varieties of wheat seeds (LSD = 2.00).

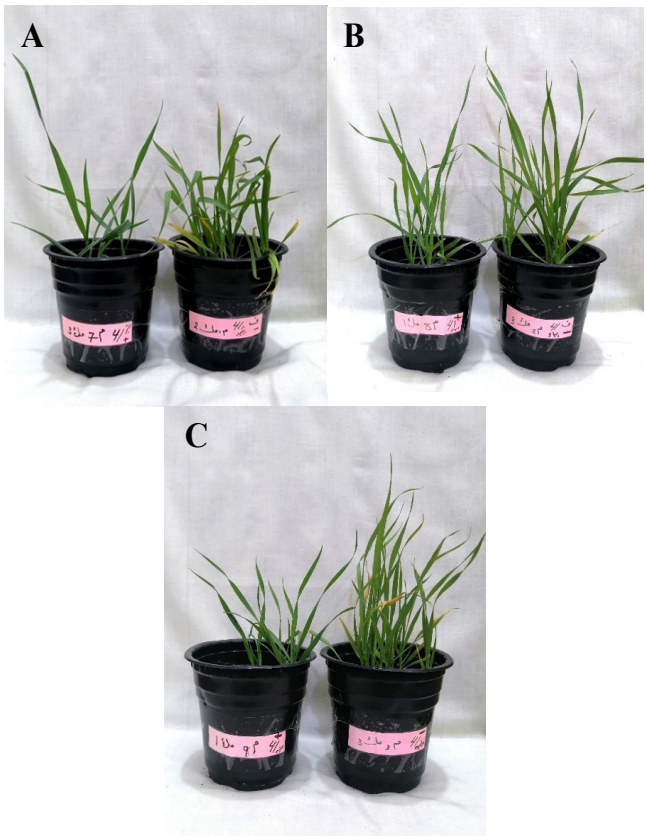


Figure 5. Effect of *A. alternata* on wheat seeds and seedlings of three wheat varieties. (A) Rashid variety, (B) Ibaa 99 variety, and (C) Wafiya variety. In each image, the left pot contains fungus-inoculated seeds, whereas the right pot served as the non-inoculated control.

Discussion

In the current study, various species of Alternaria were associated with low seed germination in several wheat fields located in different regions of Kerbala Province, Iraq. These various species included *A. alternata, A. chlamydosporigena, A. sorghi, A. cumini, A. japonica, A. tenuissima, A. hungarica, A. lolii* and *A. rosae* identified on the basis of morphological and molecular analysis. The field survey in all wheat fields revealed the presence of *A. alternata*, the main fungus isolated from wheat seeds.

A. alternata is a well-studied pathogen that can infect a variety of seeds, including those of wheat. This fungus is associated with its broad host range and the capacity to cause significant agricultural damage. In relation to wheat crop, *A. alternata* has been recognized as the primary pathogen that causes diseases on seeds, particularly the black point disease. This disease negatively affects the quality of seeds and the germination rate (Duarte *et al.,* 2022). The presence of *A. alternata* in wheat seeds can adversely affect the viability and strength of the seeds, ultimately affecting the yield and quality of the crop (Casa *et al.,* 2012).

Studies have demonstrated that *A. alternata* can adapt to survive on wheat seeds, and serve as the inoculum for subsequent crops (Casa *et al.,* 2012). The pathogen's capacity to create mycotoxins, such as alternariol, further amplifies its effect. These toxins can inhibit the process of seed germination and affect the health of young plants (Grover & Lawrence, 2017). The association of *A. alternata* with wheat seeds is not an isolated case; other plants have been infected with the same pathogen indicating its general pathogenic nature and its potential for transmission through seeds (Mangwende *et al.,* 2018). Other previously reported research has demonstrated that *A. alternata* can adapt to different environmental conditions, which increases its probability of survival and reproduction on seeds. The consequences of *A. alternata* infection are more extensive than simply the immediate seed health. Infected seeds can lead to more significant problems in agriculture, including decreased yields and reliance on pesticides for management (Mokhtar & Dehimat, 2013). As a result, understanding the behaviour of *A. alternata* in wheat seeds is pivotal in developing effective strategies to mitigate the impact of the disease in wheat production.

Molecular-based assays may facilitate accurate identification of fungi including *Alternaria* spp. in addition to morphological traits (Lahuf *et al.,* 2023; Lawrence *et al.,* 2016). However, molecular analysis faces certain hurdles, as standard genetic loci fail to distinguish Alternaria section due to minimal or nonexistent variation in molecular markers (Andrew *et al.,* 2009; Peever *et al.,* 2004; Prencipe *et al.,* 2023). Research results have indicated that the criteria for identifying low resolution in species delimitation among small-spored *Alternaria* spp. are only meaningful when a combination of different genes is utilized (Matić *et al.,* 2020). To address these challenges, Woudenberg *et al.* (2015) resolved the phylogeny of Alternaria species by employing various gene regions. Considering the current molecular analysis results, applying

several marker genes (ITS, RPB1, RPB2, TEF, beta-tubulin) have increased the discrimination ability of identification among Alternaria species (Zhang *et al.,* 2023).

The pathogenicity assay displayed that all the Alternaria species, including the isolated *A. alternata*, were pathogenic and had high virulence by causing inhibition to seed germination or entire infection to the germinated seedlings. However, in the pots assay, *A. alternata* species were less pathogenic on wheat seeds of three varieties inoculated with conidial suspension. This discrepancy may be due to the presence of different resistance genes among the three wheat varieties against *A. alternata*.

In the current investigation, it was possible for the first time to describe the diversity of Alternaria spp. associated with wheat seeds in Karbala Province, Iraq. However, further examinations are still needed to verify the presence of other Alternaria species or other fungi that could be associated with inhibition of wheat seed germination. Additionally, the genetic diversity of fungal isolates from different geographical areas related to disease should be involved in discovering the community of the pathogens of wheat seed decay. Thus, additional studies must concentrate on developing and examining active disease management approaches.

الملخص

حميد، زينب لطيف وبان طه محمد. 2026. التشخيص المظهري والجزيئي وتحليل القدرة الإمراضية وانتشار الفطر *Alternaria alternata* في حقول القمح/الحنطة بمحافظة كربلاء، العراق. مجلة وقاية النبات العربية، 44(3):320–327. https://doi.org/10.22268/AJPP-001417

يُعرف الفطر *Alternaria alternata* بأنه عامل مسبب لأمراض نباتية مختلفة في جميع أنحاء العالم. ومع ذلك، لم تتم دراسة توزيع الأنواع المسببة لفشل إنبات بذور القمح/الحنطة في العراق. خلال مسح ميداني، تمّ جمع مئة واثنتين وسبعين عزلة تعود معظمها للفطر *Alternaria* من 69 حقل قمح/حنطة في محافظة كربلاء، العراق خلال الفترة 2022–2023. بناءً على الصفات المظهرية وتحليل تسلسل الواسمات الجينية المختلفة بما في ذلك الفاصل المنقول داخلياً (ITS)، وبوليميراز الحمض النووي الريبي الموجه للحمض النووي الريبي 1 (RPB1) وبوليميراز الحمض النووي الريبي الموجه للحمض النووي الريبي 2 (RPB 2) ومورِّث عامل استطالة الترجمة (TEF) ومورِّث بيتا توبيولين (β-tubulin). فقد تم تعريف غالبية عزلات *Alternaria* التي تم الحصول عليها على أنها *A. alternata*، وعلى حدّ علمنا، يعدّ هذا أول وصف للفطر *A. alternata* باعتباره المسبب الرئيس لتحلل البذور وموت بادرات محصول الحنطة/القمح في محافظة كربلاء، بالعراق.

كلمات مفتاحية: صفات مظهرية، قمح/حنطة، القدرة الإمراضية، القرابة، *Alternaria alternata*.

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