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Short Communication

Alternaria alternata, A New Record on *Vitis vinifera* in Karbala Province, Iraq

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Abstract. Grapevine (*Vitis vinifera*) is one of the significant fruit trees cultivated in Iraq. During 2018-2019, severe leaf spot symptoms were observed commonly on grapevine in Al-Hussainiya district, Karbala province, Iraq. In order to identify the etiology of this disease, symptomatic leaf samples were collected randomly. The associated fungus was isolated, purified and identified as Alternaria alternata according to its morphological characteristics and sequence analyses of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). The pathogenicity assay using Koch's postulates approved that the isolated *A. alternata* was pathogenic on grapevine leaves. The current study represents the first record of *A. alternata* causing leaf spots on grapevine in Karbala province, Iraq.

Keywords: *Alternaria alternata*, grapevine, leaf spot, morphological characteristics

The family *Vitaceae* includes fourteen genera, including *Vitis*, the most important genus, with more than 700 species and 10,000 cultivars widely cultivated worldwide. *Vitis vinifera* L. is the most prominent of these species (Saeedi, 2000). In Iraq, 70 species and cultivars are grown the northern provinces (Abdul-Qader, 2006). Grapes have many medical and therapeutic benefits because it is considered an integrated food for what its fruits contain sugars, acids, proteins and dietary fibres, as well as being a food substance used as a stimulant for brain cells, heart muscles and an energizer for the liver and kidneys (Gamal El-Din, 2010). The global production is about 73,594,096 tons, with China leading the highest amount of production, followed by Italy and Spain (FAOSTAT, 2021). In general, grapevine in the field is exposed to infection with many fungal pathogens in pre- and post-harvest periods, leading to significant damage. These pathogens are transferred with the grapefruits to the store, where the damage increases more than its harm in the field (Al-Juboory *et al*., 2010). Some of the most significant diseases are grey mold, powdery mildew, and downy mildew (DM), caused by *Botrytis cinerea*, *Erysiphe necator* and *Plasmopara viticola*, respectively, in addition to many others (Armijo *et al.*, 2016).

Alternaria is one of those widespread fungal genera with approximately 300 species that distribute in various environments (Hameed *et al*., 2022; Lahuf *et al*., 2018; Lahuf *et al*., 2018; Lourenco *et al.*, 2009). These species are either saprotrophic or parasitic, causing various diseases on multiple plants (Hameed *et al*., 2021; Lahuf, 2019; Konstantinova *et al*., 2002). For instance, *Alternaria alternata* causes leaf spots on numerous plant hosts (Anwaar *et al*., 2022). However, the abundant number of reported *Alternaria* species has caused a complicated process distinction among them (Woudenberg *et al*., 2013). Thus, identification based on their morphological and molecular characteristics has been recommended for precise identification of them (Lahuf *et al*., 2020).

Alternaria disease on grapevine leaves increases damage rating and is one reason for reduced production. For example, in Iraq, the production of grapevines in the last three years (2019,2020,2021) is estimated at around 42,000 tons compared with 99,444 and 123,083 tons in 2017 and 2018, respectively (FAOSTAT, 2021). Thus, this study aimed to identify leaf spot pathogens that might participate in causing this production reduction.

Isolation and morphological characterization. In the season of 2018-2019, an epidemic leaf spot influencing grapevine was observed in most of Alhusaynia vineyards, Karbala province, Iraq. The symptoms of the leaf spots initially seemed like light brown small circular spots, which later developed into a dark brown irregular shape. Consequently, some of them merged to form significant

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necrotic areas and at the end of the season, the diseased leaves became yellowing and dried completely (Fig. 1).

Diseased grapevine leaves showing leaf spot symptoms were sampled randomly (100 leaf samples), cut into small segments (1-2 cm), disinfected using sodium hypochlorite solution at 2% for 2 min and washed several times *via* distilled water. These leaf sections were placed aseptically on water agar media and incubated at 25±2 °C for three days in darkness (Shehan *et al*., 2022). A hyphal tip of each emerged fungal colony was collected and placed on potato dextrose agar amended with the ampicillin and kanamycin mono sulphate antibiotics (50 µg/mL) (Jaber and Lahuf, 2020). All inoculated plates were then incubated at 25±2 °C for seven days (Lahuf *et al*., 2022). The cultural and microscopic features of the purified fungal colonies were examined and stored at 4 ºC in PDA slants for further analysis.

Molecular identification. The total genomic DNA was extracted from a week-old culture of five pure isolates utilizing the DNeasy Plant Mini Kit (Qiagen, Hilden city, Germany) (Lahuf *et al*., 2019). The internal transcribed spacer (ITS) region of the fungal rDNA was amplified using the universal primers set ITS1 (′5-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (′5-TCCTCCGCTTATTGATATGC-3′) developed previously (White *et al*., 1990). The polymerase chain reaction (PCR) was done using the ready-to-go PCR Beads kit (GE Health care, Illinois city, USA). A 21 µL of sterile double deionized water was used to dissolve a PCR Bead and 1 µL of each primer and 2 µL of the genomic DNA extracted were added and mixed thoroughly with the PCR reaction mixture. The amplification conditions of PCR started with an initial denaturation step for 5 min at 95 °C followed by 35 cycles in three steps, *i.e*., a de-naturation for 40 s at 95 °C, ana annealing for 40 s at 55°C and an extension for 1 min at 72 °C, then final extension for 5 min at 72 °C. The PCR amplicons were sequenced at Macrogen (Seoul city, south Korea) (Abdulmoohsin *et al.*, 2019). The DNA sequences produced were compared with those sequences of reference fungi at the GenBank sequence database of NCBI using the Basic Local Alignment Search Tool (BLAST) program. The phylogenetic analyses of the sequence data were performed using Molecular Evolutionary Genetics Analysis version 11 and consisted of neighbour-joining analyses. The sequence was then deposited into the GenBank database and assigned a specific accession number (Abass and Lahuf, 2022; Tamura *et al*., 2021).

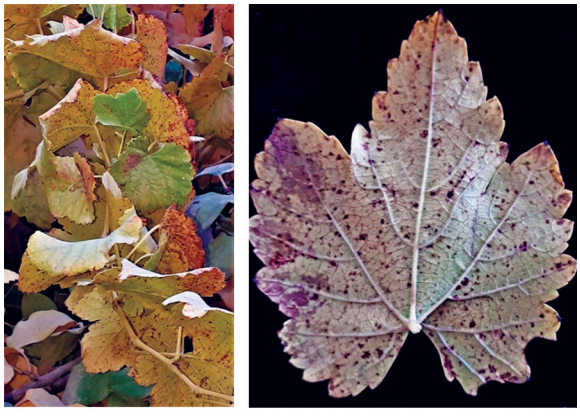

Fig. 1. Leaf spot symptom on grapevine leaves in the Alhusaynia vineyards.

Pathogenicity assessment. Finally, the pathogenicity ability of the isolated fungus was assessed using the detached leaf assay. Healthy grapevine leaves were surface disinfected with Ethanol (70%) for 30 seconds, and each was inoculated with 500 µL of the pure fungal conidia suspension at a concentration 1x106 conidia/mL. The fungal conidial suspension was prepared by adding 20 mL of sterile distilled water to the fourteen day pure sold culture and harvesting the fungal conidia. The suspension was then poured through a sterile double layer of cheesecloth to eliminate residual Agar and Mycelia. The conidial suspension was then prepared with 0.05% Tween® 80 (Sigma Aldrich Co, USA) and diluted to 1 × 106 conidia/mL aided *via* a hemocytometer (Hirchmann Laborgeräte, Eberstadt, Germany).

On the other hand, the control leaves were treated with sterile distilled water only. Subsequently, leaves were incubated in a growth chamber at 25±2 °C and monitored daily. After 7 days, the leaf spot symptoms appeared. Then, the pathogenic fungus was re-isolated after 14 days of inoculation and identified morphologically and molecularly (Akhtar *et al*., 2011).

Numerous fungal isolates were associated with the symptomatic leaves of grapevines. However, in most of them (78 out of 100 leaf samples), the mycelial growth of these fungal colonies on PDA media was consistently a greyish-white radial, airy, compressed and cottony-like shape that converted latterly to dark olivaceous with the same structure (Fig. 2 A).

This cultural appearance was consistent with almost all the colonies obtained. The fungal conidiophores were

short, curved in a simple form, carrying numerous conidia. These conidia were oval to pyriform shape in dark brown colour with transverse (1–3) and longitudinal (0–2) septate. They were in average length and width, extended from 15 to 35 µm and 8 to 12 µm, respectively. They were usually solitary and hardly seen in chains structure (Fig. 2 B). These cultural and microscopic appearances, which agreed with previous descriptions (Simmons, 2007; Lahuf, 2019), proposed that the fungus isolated was likely one of the *Alternaria* species. This initial identification was confirmed via the Basic Local Alignment Search (BLAST; Fig. 3) and the phylogenetic analysis (Fig. 4). of the isolate sequence (GenBank Accession No. MK069992.1) that displayed an almost identical similarity (99.74%) with multiple international strains of *A. alternata* such as KJ191593.1, OQ064058.1 and OQ055256.1.

After one week of *A. alternata* inoculation, small light brown circular spots (Fig. 5). appeared on the inoculated leaves and some of them expanded and merged to form larger infected areas approximately 2-10 mm in width. On the other hand, control leaves were symptomless. Therefore, the accompanying pathogenic fungus, *A. alternata,* was re-isolated from all of the symptomatic leaves of grapevines and re-identified, relying on its morphological and molecular characteristics, which were comparable to the original isolate of *A. alternata*.

The fungus *A. alternata* was recorded as a causal agent of different diseases, including the leaf spot on diverse plant hosts. For example, it was reported on *Rumex*

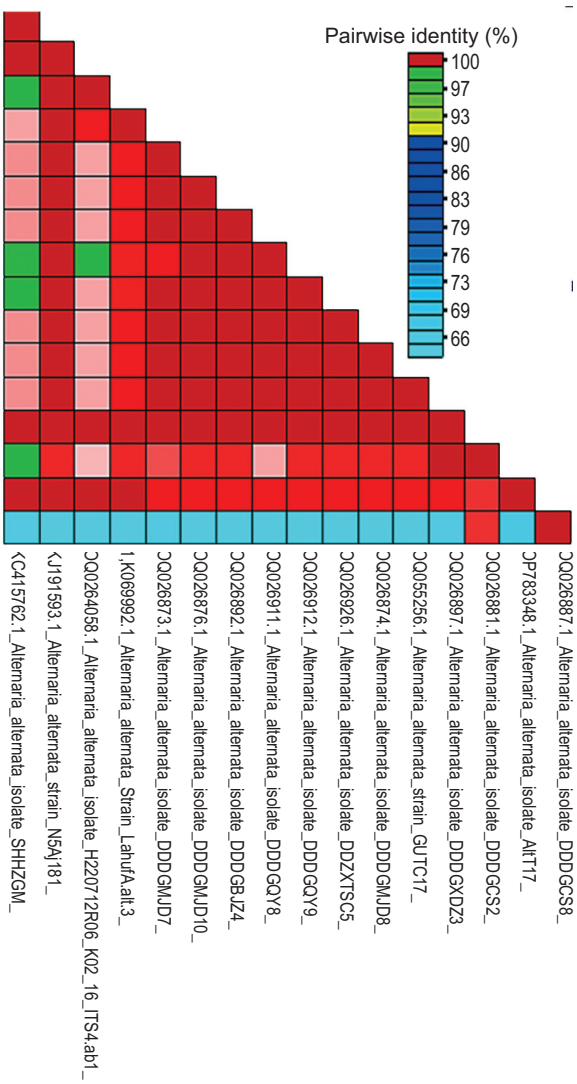

Fig. 3. Pairwise similarity scores of the isolated fungus (indicated by a red rectangle) with other global *A. alternata* isolates and strains display a colour-coded matrix. This diagram was based on the BLASTn analysis results.

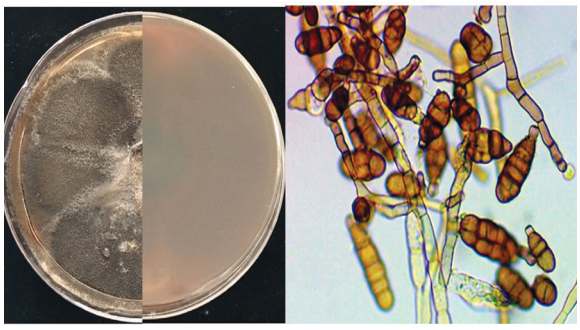

Fig. 2. Cultural and microscopic characters of *A. alternata* associated with leaf spots on grapevines; **(A)** The upward (left) and downward (right) surfaces of *A. alternata* culture on PDA medium; **(B)** Conidia of *A. alternata* conidia. The scale bar is 20 µm.

vesicarius, *Musa* sp.; *Cydonia oblonga*, *Prunus armeniaca* and *Ficus carica* (Hameed *et al*., 2021; Lahuf *et al*., 2020; Parkunan *et al*., 2013; Sankar *et al*., 2012). Nevertheless, there is no previous description regarding *A. alternata* causing the leaf spots on grapevine in Karbala, Iraq.

Conclusion

Grapevine (*V. vinifera* L.) is one of the most important fruit crops worldwide. Hence, determining those biotic

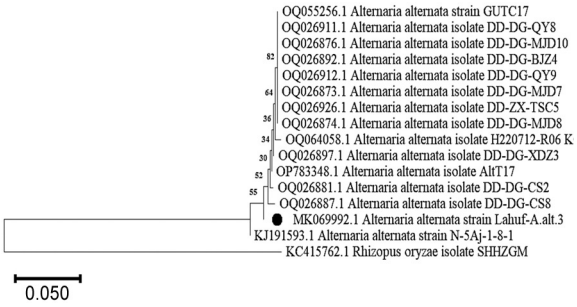

Fig. 4. Phylogenetic tree of the *A. alternata* isolated in this study (indicated with a black dot) with other global *A. alternata* strains deposited in the GenBank database. This phylogenetic tree relied on the BLASTn analysis that displayed the relationship among the isolated *A. alternata* and other universal strains or isolates of the same fungus.

Fig. 5. Symptoms of leaf spots on the inoculated grapevine leaf.

and abiotic stresses that reduce the quality and quantity of this crop is essential. The aetiology of an epidemic leaf spot on grapevine in Alhusaynia vineyards, Karbala province, Iraq, during the season of 2018-2019. The causative agent was identified as *A. alternata* based on its morphological and molecular features. To our knowledge, this is the first report of leaf spots caused by *A. alternata* on grapevine in Karbala, Iraq. Therefore, further studies and surveys should be undertaken in other Iraqi grapevines to unveil the leaf spot disease incidence and severity and determine the best practices to control it.

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Conflict of Interest. The authors declare they have no conflict of interest.

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