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FIRST RECORD OF *DACTYLONECTRIA ALCACERENSIS* THAT CAUSE GRAPEVINE BLACK FOOT DISEASE IN IRAQ

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ABSTRACT

Two pathogenic isolates of the genus *Dactylonectria* associated with black foot disease of grape rootstocks (2-3 years old) were identified in Duhok province, Iraq. The disease incidence recorded 67% on plants that lead to weaken or plant failure when disease severity exceeded 51%. This disease is characterized by black or dark brown necrotic lesions at the bottom of rootstocks, sparse foliage and plant growth reduction. The molecular identification of CD8 and CD9 isolates, revealed that they were 100% identical to *D. alcacerensis*, both isolates were deposited in the national center for biotechnology information (NCBI) under accession numbers ON129803 and ON129804 respectively.

***KEYWORDS*:** *Cylindrocarpon* sp., Morphology, PCR, Phylogeny, ITS

INTRODUCTION

Grape *(Vitis vinifera)* is an economically important subtropical fruit tree cultivated in Kurdistan region, Iraq. They are also cultivated in Mediterranean region, central Europe, and southwestern Asia (Singh & Chauhan, 2020). Black Foot Disease (BFD) is a major disease that attacks grapevine rootstocks in nurseries across the world. During 2018-2022 BFD has become interesting problem in different middle –east and western Asia countries including Iraq (Gungor-Savas *et al.*, 2020; Najim El-din & Haleem, 2022). A wide variety of *Cylindrocarpon*-like anamorphs were recorded as pathogens for this disease (Gramaje *et al*., 2018).

On vines that were 2-3 years old, BFD symptoms included a decline in plant vigor, discoloration, and vascular necrosis at the base of the plant stem. Streaking of vine vascular tissues, xylem vessel gum inclusion, dieback, decline of young grapevines, weak sprouting, and bud burst disorder may also be observed (Langenhoven *et al*., 2018).Colonies of the isolates ranged from orange to dark brown. Sub cultures on PDA form hyaline, cylindrical, straight, and/or slightly curved, 1-3 septate macroconidia and numerous hyaline, oval, one-septate microconidia (Gungor-Savas *et al*., 2020).

According to Urbez-Torres *et al*.(2012) and Najim El-din & Haleem (2022), *Cylindrocarpon* species may be diagnosed molecularly using ribosomal internal transcribed spacer (ITS) region with primer pairs ITS1 and ITS4.According to our knowledge, the current work could be considered as the first record of *Dactylonectria alcacerensis* in Iraq viticulture.

MATERIALS & METHODS

Field survey

To determine severity and frequency of (BFD), which affected commercial nurseries and horticulture stations between September and November 2021, four grapevine nurseries in Duhok, Iraq, were surveyed. The commission of the European Community (CEC) standard method of plot assessment was applied. According to (Alaniz *et al*., 2007), disease severity was evaluated utilizing a scale of 0 to 5 depending on the degree of black discoloration.

Disease severity was estimated based on the formula of Alaniz *et al*., (2010). Reduction of plant vigor on the basis of shoots elongation and decrease in root mass as a result of pathogens aggression was recorded. The former was calculated utilizing the formula below (Hassan *et al*., 2013):-

$$\%\text{Reduction} = \frac{\text{Whr} - \text{Wdr}}{\text{Whr}} \times 100$$

Where: Whr = Weight of healthy root,

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Wdr = Weight of diseased root

Plant materials & Fungal Isolation

Samples were collected from symptomatic rootstocks at root elongation. According to Agusti-Brisach & Armengol (2013), 5-10 samples from roots, callus and lower nodes were randomly taken from four separate nurseries. Grapevine samples were disinfected using 2% NaOCl and then gently washed with sterile D.W. prior culturing on (PDA supported with 250 mg L^{-1} chloramphenicol) at 25 ± 2^0 C.

Morphological diagnosis

According to Haleem *et al*.(2014), isolates were grown on PDA at 25+2°C in darkness for 20 days, followed by near ultraviolet (NUV) with a 12-h light / dark cycle for 10 days for cultures sporulation. Fungal isolates were identified morphologically based on the characters described by (Damsch *et al.*, 1980; Petit & Gubler, 2005).

Molecular identification

DNA extraction, PCR amplification, and Sequencing

Genomic DNA was isolated from fungal isolates utilizing DNA preparation kit (Jena Bioscience GmbH.07749 Jena, Germany).

PCR amplification for DNA was performed in 50 µl reaction mixture included: 25 µl of 2x Taq DNA Polymerase Master Mix, 2 µl of ITS1 primer (5'- TCCGTAGGTGAACCTGCGG -3'), 2 µl ITS4 primer (5'- TCCTCCGCTTATTGATATGC -3'), the same primer used for the first time by White *et al*. (1990), 17 µl DNase free water (Ultrapure free distilled water) and 4 µl DNA template the PCR reaction was carried out by Bioresearch PTC-200 Gradient thermo-cycler. Temperature profile involved at 95^0 C for 5 min, 35cycle at 95^0C for 1 min, 55^0C for 60 seconds, 72^0C for 1 min and final step at 72^0C for 5 min. PCR products visualized using 1% agarose gel stained with 3 µl of Eva-Green® Fluorescent Gel Stain. ABI Prism Terminator Sequencing kit (Applied Bio system) was used to sequence the PCR products of fungal isolate specimens at Macro gene Company in South Korea, Chromatograms were edited and base calls checked using Finch TV 1.4.0 program software developed by (Geospiza Inc., Seattle WA,USA) according to the principles outlined by (Alachiotis *et al*.,2013).

The resulting sequences were exposed to Basic Local Alignment Search Tools (BLAST), to compare sequences with the NCBI deposited sequence. There identified sequences were submitted to the National Center for Biotechnology Information (NCBI).

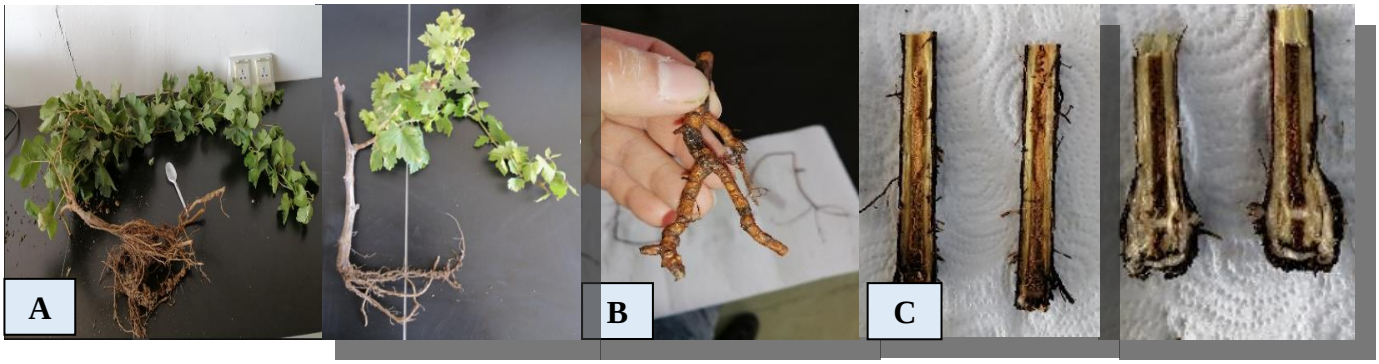
Phylogenetic analysis

To ensure the accuracy of the DNA sequences, the results were deposited in Bio-Edit version 7.The BLAST tool was used to identify similarities between the sequences produced during this experiment and other sequences in Gen-Bank. Cluster method (iteration 1, 2 and other iteration) utilized to make the comparison between chosen sequences. Maximum parsimony method utilized to construct evolutionary history of *Dactylonectria alcacerensis* in MEGA.11 and bootstrap consequence tree built with 1000 replicates. Twenty one nucleotides sequences were included; chosen codon composition involved 1st + 2nd + 3rd + noncoding sites. *Campylocarpon fasciculare* used as out-group.

RESULTS & DISCUSSION

Field survey

Disease incidence and severity were assessed on rootstocks depending on diagnostic symptoms of brown discoloration at bottom of rootstocks and reduced plant growth as shown in (Fig.1). These evidences were similar to reports of (Vicent, 2019; Yildiz & Tosun, 2022).The highest disease incidence 67.5% and disease severity 51% were recorded in horticulture station nursery on Zaetony cv. In commercial nursery 1, Tabrreezy cv. shows more susceptibility to disease and reduction of shoots growth attained to 63.39%.In the same location, the root growth of Zaetony cv. was also restricted to 54.2%. Rashmery cv. in commercial nursery 2 revealed less sensitivity to BFD due to disease incidence 45.5% and disease severity 38%.Thus, their roots growth was reduced by 11%.The virulence of *Cylindrocarpon* species contribute to their ability to secreting of oxidative pectolytic enzymes that weaken plant cell walls enabling pathogens penetration (Kikot *et al*., 2009; Probst *et al*., 2019), and their ability to colonize plant tissues after infection, as well as the host plant's susceptibility (Cabral *et al*., 2012b; Bleach *et al*., 2021).



Morphological Characterizations

The results revealed that colonies diameter of a pathogen was (66.5-68.5 mm). The colony's center was orange with rough form. Small sporulation core with a flat, buried violet color inner border and coral outside edges (Fig.2-A, B).

Macroconidia (34.6 × 5.3 µm), segmented with 1-3 septa, colorless, cylindrical, erect and/or gently bent. Microconidia were transparent, elliptical, unseptate or with one septum (12.5 × 6 µm) and no chlamydospores. (Fig.2-C). Morphological characteristics of our samples; Cultures and sporulation were similar to *Ilyonectria* genus (Cabral *et al*., 2012a; Lombard *et al.*, 2014; Yildiz & Tosun, 2022).

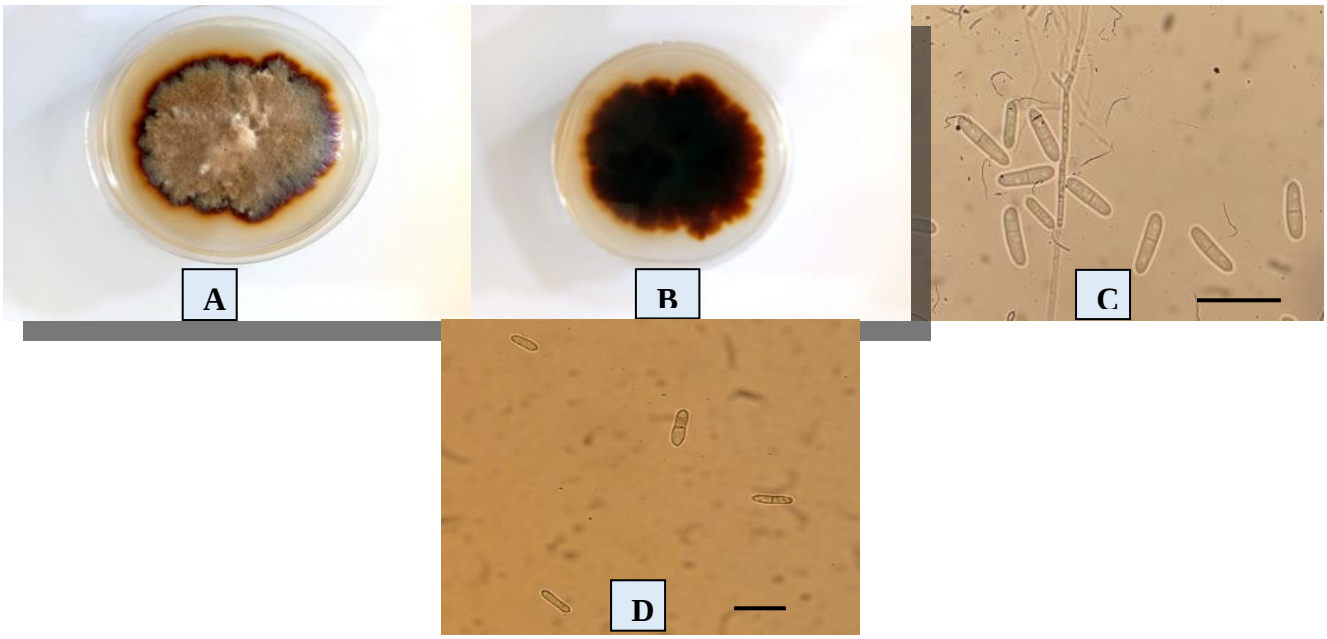


Fig. (2) Colony of *Dactylonectria alcacerensis* (A-upper view, B-lower view, C- Macroconidia, and D-Microconidia, Scale bar C =40 µm , D= 20 µm)

Sequencing & Phylogenetic Tree

The morphological diagnosis was confirmed molecularly. The sequence comparison with the deposited sequence in NCBI database confirmed that each isolate of CD8 (ON129803) and CD9 (ON129804), have been 100% compatible with the *Dactylonectria alcacerensis* (A. Cabral, Oliveira and Crous) L. Lombard & Crous.

The length of the rDNA-ITS sequence of *D. alcacerensis* isolates ranged between 470-480 bp of ITS1-ITS4. Phylogenetic analysis revealed that the attained sequence shares 100% similarity to *D. alcacerensis* strains: China isolate (MN988716), and USA isolate (MK400306) (Fig. 3).These findings were accordance with several authors (Mora-sala *et al*., 2018; Berlanas *et al*., 2020; Ye *et al*., 2021).

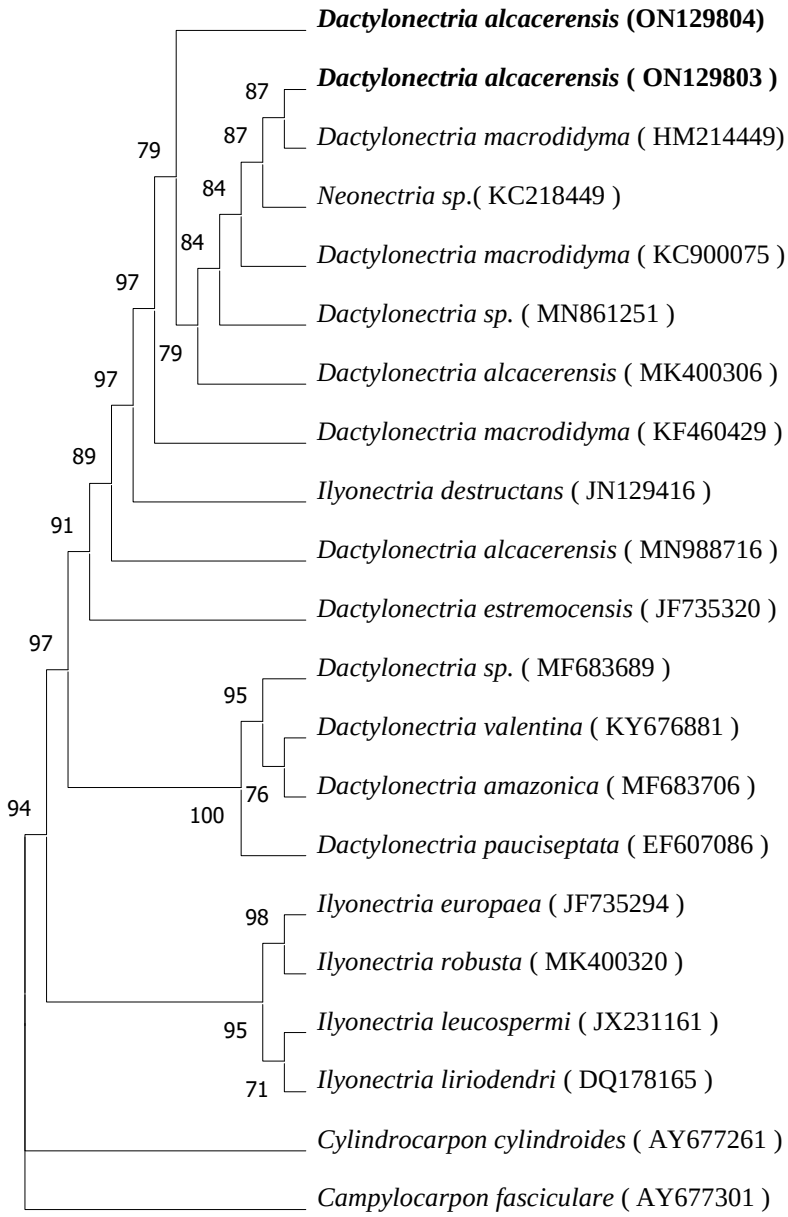


Fig. (3):- Evolutionary analyses were conducted in MEGA.11, employing maximum parsimony method of ITS sequences. Blast shows phylogenetic positioning of *Dactylonectria alcacerensis* with 1000 bootstrap. *Campylocarpon fasciculare* used as out-group. Studied isolate from Iraq in Bold. Gen-bank accession numbers come after species name

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CONCLUSION

In the current work, we sequenced the ITS region of *D. alcacerensis* from symptomatic grapevine rootstocks. Sequence in Gen-Bank confirming the identity of both examined isolates and phylogenetic tree made based on this information. Therefore, *D. alcacerensis* was recorded for the first time on grapevine rootstocks in nurseries of Duhok, Iraq.

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تومارکرنا Dactylonectria alcacerensis ئەگەرێ نەخوشیا پنیێن رەش ل سەر تری بو جارا ئیکێ ل عێراقێ

پوختە

ئەڤ ڤەکولینە هاتە ئەنجامدان ژ بو دیارکرنا دوو ئایزولەیتێن کەروی ژ جنسێ Dactylonectria ئەوێ دبنە ئەگەرێ پەیدابونا پنیێن رەش لسەر نەمامێن تری ب ژیێ 2-3 سال ل پارێزگەها دهوك/ عێراق. نەخوشی دیاربو برێژا 67% و بو ئەگەرێ لاواز بون و مرنا نەمامان پشتی توندیا نەخوشیێ گەهشتیە 51% ل پارێزگەها دهوك. نەخوشی هاتە دیارکرن بریکا دیتنا پنیێن رەش و قەهوائی ل سەر رهێن نەمامێن تری و کێمبونا ریژا کەسکاتییا رووەکی. ناسناما هەردو ئایزولەیتا CD8 و CD9 ب کودێ جین بەنك (ON129803 و (ON129804 هاتە دیارکرن بریکا PCR و د ئەنجامدا دەرکەفت کو هەردو ئایزولەیت 100% وەك هەڤن دگەل Dactylonectria alcacerensis.

أول تسجيل ل Dactylonectria alcacerensis المسبب المصاحب لمرض القدم ألأسود للعنب في العراق

الخلاصة

الهدف من الدراسة الحالية هو الكشف عن إثنين من عزلات *Dactylonectria* الممرضة المرتبطة بمرض القدم السوداء في جذور العنب(2-3 سنوات) في دهوك/العراق.ظهر المرض بنسبة 67% والتي أدى الى ضعفها وفشل معظمها بعد وصول شدة المرض الى 51% في محافظة دهوك،العراق. شُخص المرض من خلال أعراض التنخر الأسود و البني الداكن لقواعد الشتلات، وقلة المجموع الخضري و اختزال نمو النبات. اجرى تشخيص المرض جزيئيا لكلتا عزلتي الفطر CD8و CD9 لتحديد نوع العزلتين و أظهرت النتائج تشابها عاليا بنسبة 100% مع *D. alcacerensis* (ON129803 و ON129804) لكلا العزلتين علئ التوالي.