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CHARACTERIZATION AND REPORT OF *ALTERNARIA ALTERNATA* CAUSING LEAF SPOT DISEASE IN PLUM TREES IN KARBALA IRAQ

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A B S T R A C T

In the growth season of 2018-2019, plum trees in many orchards in the Al-Hussainiya district of Karbala Province, Iraq, regularly exhibited severe symptoms of leaf spot. The prevalence of the disease was estimated to be 15%. The causal factor responsible for this disease has been determined as *Alternaria alternata* based on its morphological and molecular characteristics. The pathogenicity of the isolated *A. alternata* was assessed using Koch's postulates, which confirmed its pathogenic nature. Therefore, it was once again isolated from the affected symptomatic leaves. The results outline the scientific methodology used to identify the causal agent responsible for the Alternaria leaf spot disease on plum trees. This is the first report of *A. alternata* causing leaf spot on plum trees in Karbala province, Iraq.

Keywords: Leaf spot, *Alternaria alternata*, rDNA-ITS and TEF-1α sequencing, Plum tree.

INTRODUCTION

Plum tree (*Prunus* sp.) is a small deciduous tree that belongs to the family Rosaceae, which is believed to be native to China (Bortiri *et al.,* 2006; Okie and Hancock, 2008). The main purpose of culturing of this tree is to costume its fruits that are characterized by containing carbohydrates, protein, fiber and vitamins such as vitamins A, B, and C. besides some organic acids such as citric and malic. It is consumed directly or used for many purposes such as making jams (Sanders, 1993; Lozano *et al.,* 2009; Voca *et al.,* 2009). At present, this tree grows worldwide in different countries such as China, Romania, Serbia, Iraq etc. Annually, Iraq produces only 16, 584 metric tons of plum, which made it ranked 45th among the countries participating in global production (FAOSTAT, 2023). This relatively low production percentage of plum in Iraq is due to affect numerous pests such as bark beetles, Aphids and fruit fly besides many diseases including the root rot, powdery mildew, and rusts causing a significant reduction of the quality and yield to the plum trees (Kole and Abbott, 2012).

Alternaria genus comprises diverse saprophytic and pathogenic species. *A. alternata*, *A. tenuissima*, *A. brassicae*, *A. brassicicola*, *A. radicina*, and *A. infectoria* are the supreme *Alternaria* pathogenic species. They affect widespread economically vital plants, including Apples, Grapes, Oranges, Lemons, Melons, Tomatoes, Cucumbers, and Peppers in the pre-postharvest time (Hussein *et al.,* 2019; Ramezani *et al.,* 2019) that have caused significant losses as a result of fruit and vegetable rot (Meena *et al.,* 2017; Pinto *et al.,* 2017). Lately, this genus has been divided into 26 sections based on morphological features (Woudenberg *et al.,* 2015). For instance, spore isolates of small size were found in the majority of sites studied, accounting for almost 80% of all Alternaria isolates. The isolates belonging to this category were primarily identified as *A. alternata* and *A. tenuissima*. However, the spore isolates of significant size were determined to be *A. linariae*, *A. solani*, and *A. grandis*, comprising less than 20% of the total Alternaria species present. However, The ITS marker sequencing was found to discriminate effectively the *Alternaria* spp. that exhibited similar morphology (Saleem *et al.,* 2022). Several studies identified precisely the *Alternaria* sp. causing the diseases

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in different plant hosts using this marker. For instance, Parkunan *et al.* (2013) found that *A. alternata* was the pathogen of the Alternaria leaf spot of banana. Moreover, Lahuf (2019a) and Lahuf *et al.* (2020a) identified that *A. alternata* and *A. solani* caused the blight disease on rosy periwinkle and Tomato plants respectively. However, recently it has been found that the identification of *Alternaria* spp. using more than one marker sequences is more reliable and effective in the accurate identification of this genus member. For example, Lahuf *et al.* (2020b) applied three markers the internal transcribed spacers (ITS) region, translation elongation factor 1-alpha (tef1), and Actin (actA) genes for identification *A. alternata* that infect quince trees and cause leaf spot disease.

This study aimed to determine the specific agent responsible for the leaf spot disease in plum trees in the orchards of Al-Hussainiya district in Karbala Province, Iraq.

MATERIAL AND METHODS

Isolation and morphological identification of the pathogen: A large number of plum leaf samples exhibiting severe leaf spot symptoms were gathered during a survey conducted in 2018-2019 from the orchards in the Al-Hussainiya area of Karbala Province, Iraq. The disease symptoms initially manifested as little circular spots with a light brown color, which later developed into an irregular shape with a blackish brown color. However, a few of these spots maintained a circular shape with concentric spots. As a result, these spots merged to create significant areas of dead tissue, ultimately causing the leaves to become dry and yellow (Figure 1). The leaf samples were carefully washed with tap water and then treated with a 1% (w/v) solution of sodium hypochlorite (NaOCl) to sanitize the surface. Afterward, the leaves were cut into pieces measuring 1-2 cm in length (Lahuf *et al.,* 2018a). Following the drying process with sterilized filter paper, the samples were then positioned on water agar (WA) plates and subjected to incubation at a temperature of 25°C in the absence of light for a duration of three days (Lahuf, 2019b). The tip of each developing colony's hypha was taken and put onto potato dextrose agar (PDA) plates that contained amoxicillin at a concentration of 200 µg/ml. Subsequently, all plates were placed in an incubator set at a temperature of 25 °C and kept in darkness for duration of one week. Following the purification of fungal colonies, a thorough examination was conducted to assess the morphological characteristics of the pure fungal cultures, including the mycelial structure and color. The conidia (n=50) of the fungal isolates were also examined and measured (Almousawy *et al.,* 2017; Lahuf *et al.,* 2022).

A B

Figure 1. Symptoms of leaf spot on plum (*Prunus* sp.). (A) the disease symptoms on a plum tree. (B) the disease symptoms on leaves of plum.

Pathogenicity assessment: To assess the pathogenicity of the fungal isolates, a detached leaf assay (Pettitta *et al.,* 2011) was conducted using healthy leaves of plum that were surface sterilized for 30 s using 70% ethanol. Subsequently, they were placed onto a sterile filter paper inside a plastic container. Afterwards, each individual was injected with a 0.5 cm disk containing only fungal colony. Alternatively, control leaves were inoculated solely with fungal-free discs (Al-Tememe *et al.,* 2019). The leaves that were inoculated were placed under airtight lids in boxes to maintain a high level of humidity. They were then incubated at a temperature of 25 ± 2°C for a period of one week. Each replication consisted of six leaves (Lahuf, 2019a). To satisfy Koch's postulates, the fungus that was linked to the symptoms was isolated again from the affected leaves and analyzed using the same methods as before. Nevertheless,

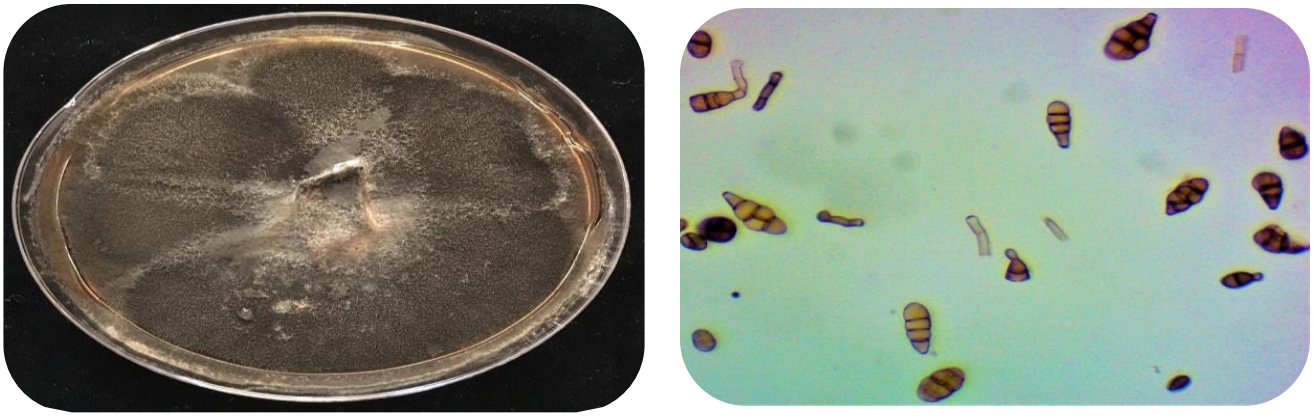
the control leaves remained free from infection.

Molecular identification of the pathogen: The complete genomic DNA was obtained from the fungal isolates that were pure and had been cultured for 7 days. The extraction procedure used was the same as the one disclosed in a previous study by Lahuf *et al.* in 2019. The Polymerase Chain Reaction (PCR) assay was conducted to amplify the Internal Transcribed Spacer (ITS) using the universal primers ITS1 (5`-TCCGTAGGTGAACCTGCGG-`3) and ITS4 (5`-TCCTCCGCTTATTGATATGC-`3). The PCR reaction conditions began with an initial denaturation phase at 94°C for 5 minutes, followed by 35 repeating cycles consisting of three steps: denaturation at 94°C for 30 seconds, annealing at 56°C for 40 seconds, and extension at 72°C for 45 seconds. Next, perform a final extension step at a temperature of 72° C for a duration of 5 minutes, as described by White *et al.* (1990) and Lahuf (2019a). In addition, the Translation elongation factor 1-alpha gene (TEF-1α) was amplified using the primer pair EF1-728 F (5`-CATCGAGAAGTTCGAGAAGG-`3) and EF1 986R (5`-TACTTGAAGGAACCCTTACC-`3). The PCR protocol consisted of an initial denaturation phase at 94 °C for 3 minutes, followed by 40 cycles consisting of three steps: denaturation at 94 °C for 30 seconds, annealing at 52 °C for 30 seconds, and a final extension at 72 °C for 45 seconds (Carbone and Kohn, 1999; Lahuf *et al.,* 2020b). The PCR amplicons underwent sequencing at Macrogen, located in Seoul, South Korea, using the ABI 3730xI automated Sequencer manufactured by Applied Biosystems.

The analysis of chromatograms and nucleotide sequences obtained from sequencing was conducted using Chromas software version 2.6.4 (Abdulmoohsin *et al.,* 2019). Subsequently, the acquired sequences were compared to the GenBank sequence database at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). The Molecular Evolutionary Genetics Analysis (MEGA) Version 10.1.5 software was used to analyze the sequence data and construct a phylogenetic tree. The Neighbour joining method was employed using the marker's sequences obtained in this study and others recorded in the NCBI. The phylogenetic tree was constructed based on the Tamura *et al.* (2013) and Jaber and Lahuf (2020) studies. The modified sequences of the detected fungal pathogen were submitted to the NCBI-GenBank Database for documentation.

RESULTS AND DISCUSSION

Isolation and morphological identification of the associated fungus: A fungus was constantly obtained from the diseased leaves of plum trees. The cultural growth of this fungus was rapidly developing wooly blackish olivaceous green colonies (Figure 2 A). The microscopic examination revealed that the conidia shapes (Figure 2 B) were obclavate and muriform with a short narrowed or tubular pale beak and had 1-3 transverse septa with 0-2 longitudinal or slanted septa. The mean width and length of these conidia (*n*=50) were 12 µm and 22 µm respectively. Depending on these morphological appearances, which were comparable to previous descriptions (Ellis, 1971; Simmons, 2007), the fungus associated with the symptomatic plum leaf was primarily recognized as one of *Alternaria* spp.



A B

Figure 2. Cultural and microscopic morphological characteristics of *A.alternata* isolated from diseased leaves of plum trees. (A) Pure colony of *A.alternata* grown on PDA; conidia of *A.alternata*. Scale bar is 20 µm.

Pathogenicity assessment: After 3 weeks, the disease incidence of the inoculated plum leaves was 100% (Figure 3) while the non-inoculated leaves were disease symptom-less. From each diseased leaf, the infectious agent, *Alternaria* sp., was re-isolated, and its microscopic characteristics were identical to those of the original isolates. This led to the successful application of Koch's postulates.

Figure 3. Detached leaf with disease symptoms of *Alternaria* sp.

Molecular identification of the pathogen: The PCR-amplified products of the ITS-rDNA and TEF-1α markers were effectively sequenced. The sequences were documented at the NCBI-GenBank under the accession numbers OP656314 and OP677548, correspondingly. The BLAST analysis of these marker sequences revealed a high identity (100%) with the corresponding globally recognized sequences of *A. alternata*. The phylogenetic analysis validated this identification by clustering the sequences of both markers from the acquired isolate with multiple reference isolates of the fungus *A. alternata* (Figures 4 and 5). Therefore, according to these findings, it was conclusively determined that the fungus is *Alternaria alternata* (Fr.) Keissl.

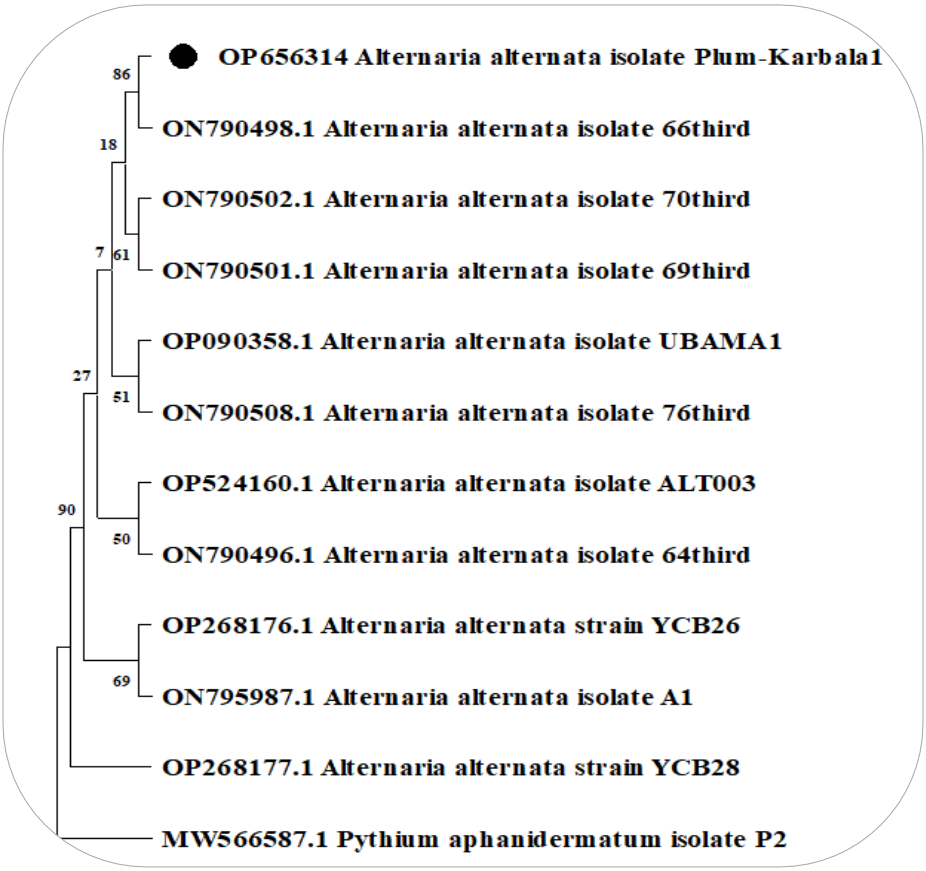


Figure 3. A phylogenetic tree was created using the ITS-rDNA sequence of *A. alternata* acquired from the infected plum leaves in this study (identified by a black dot), as well as additional strains and isolates of the same species downloaded from GenBank-NCBI.

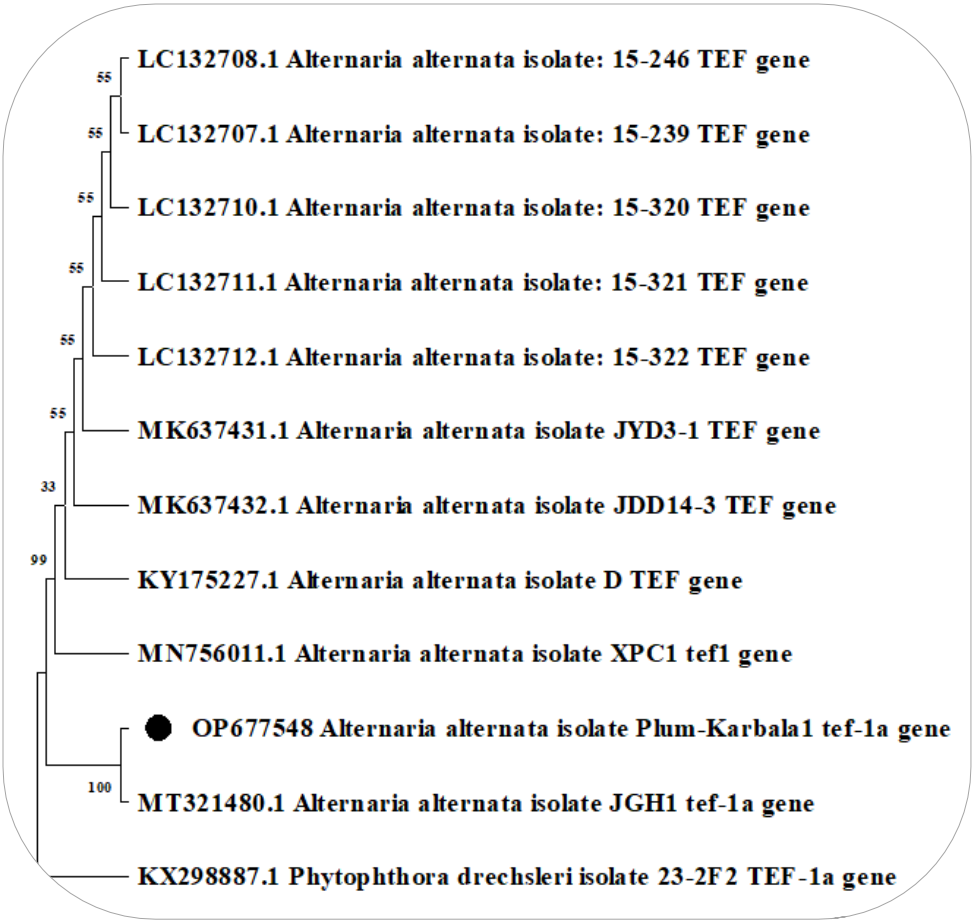


Figure 4. Phylogenetic tree built utilizing the TEF-1α sequence of the *A. alternata* gained from the diseased plum (determined by black dote) and other strains and isolates of the same species collected from GenBank-NCBI. The out of group fungus was *Phytophthora drechsleri* isolate 23-2F2

The present investigation has indicated that *A. alternata* is the causative agent associated with leaf spot of plum trees in the orchards of Al-Hussainiya district in Karbala Province which is considered one of those important agricultural regions in Iraq for planting various fruit trees such as orange, pomegranates, grapes, date palms, and plum. The identification of the organism was determined by analyzing its morphological and molecular properties, as well as evaluating its pathogenicity.

In fact, numerous studies were reported *A. alternata* infecting various plants causing different diseases such as leaf blight on rosy periwinkle (Lahuf, 2019a) and lily (Lahuf *et al.,* 2018b) as well as leaf spot on quince trees (Lahuf *et al.,* 2020b); apricot trees (Hameed *et al.,*2021) and plum trees (Long *et al.,* 2021). Although *A. alternata* is a phytopathogen predominant internationally infecting widespread plant host, it has not been recorded in Iraq particularly in Karbala province infecting plum trees. To our knowledge, *A. alternata* causing leaf spot on Plum trees *(Prunus* sp.) is first report in Karbala, Iraq.

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Hayder Abdulhasan Ali	:	Collected the data and contributed to data analysis
Abdulzahra J. Ali	:	Designed the experiment
Adnan A. Lahuf	:	Performed the analysis and wrote the paper
Duraid K. A. Al-Taey	:	Collected the data