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RAPID COMMUNICATION

Isolation and characterization of *Alternaria malorum* as a causal agent of bark canker on walnut trees

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Abstract

During 2016–2020, a longitudinal bark canker was observed on walnut branches in some of the provinces of Iran. The symptoms appeared on one side of the branches. No visible symptoms were observed on the sapwood after removal of the bark using a blade. In order to detect a potential agent of these symptoms on walnut trees, collected samples were transferred to the laboratory for further investigation. After isolation and purification based on standard methods, a fungus was frequently isolated from symptomatic tissues. Morphological and molecular assays indicated that the responsible agent of this disease was *Alternaria malorum,* moreover, a pathogenicity test confirmed that *A. malorum* was pathogenic on walnut trees. To the best of our knowledge, this study represents the first attempt to identify *A. malorum* as a new causative agent of bark canker on walnut trees in the world.

Key words: *Chalastospora gossypii*, *Cladosporium malorum*, fungal pathogen, *Juglans regia*

Walnut (*Juglans regia*, Juglandaceae) is one of the most valuable nut crops in the world. Iran is one of the largest walnut crop producing countries worldwide (Sohrabi and Mohammadi 2020). During 2016–2020, walnut orchards in the main walnut-growing provinces of Iran including Chaharmahal and Bakhtiari, Esfahan, Hamedan, Kerman, Kermanshah, Kohgiluyeh and Boyer-Ahmad and Kurdistan were surveyed for investigation of canker symptoms on walnut trees. For this purpose, samples with longitudinal bark canker were collected and transferred to the laboratory for further investigation (Table 1). The canker symptoms were developed on one side of the branches (Fig. 1A). No visible symptoms were observed on the sapwood (Fig. 1B). In order to identify the fungal agent associated with bark canker symptoms on walnut trees, fungal isolates were obtained by inserting small pieces (5 mm^2, four pieces in each plate) from the border of symptomatic and healthy tissues on potato dextrose agar (PDA) medium (200 g potato, 20 g dextrose, 15 g agar, 1,000 ml distilled water) after surface sterilization by immersing them in 1% sodium hypochlorite for 2 min, rinsing in sterile water for 3 min and drying on sterile filter paper. The culture plates were incubated at 23–25°C in the dark until fungal growth. After purification, fungal isolates were screened for morphological characterization. Preliminary identification was based on Barnett and Hunter (1972). Purified isolates were transferred to potato carrot agar (PCA) and kept at 23–25°C under a cool white fluorescent light with an alternate 8 h light/16 h darkness for 5–7 days (Bagherabdi *et al.* 2017). Microscopic observations and the dimensions of 50 randomly selected conidia and conidiophores were recorded using a BX53 microscope (Olympus, Tokyo, Japan) equipped with Nomarski differential interference optics. Fungal isolates were identified on the basis of the following morphological and cultural characteristics (Goetz and Dugan 2006). Colony color was olive-brown and gray at the center with a white margin on PCA (Fig. 2A). Conidiophores were olive–brown, simple, short, aseptate and 10–17 µm × 2–4 µm in size (Fig. 2B). Conidiophores produced long branched chains of olive-brown, aseptate, cylindrical and *Cladosporium*-like conidia,

Table 1. Details of obtained isolates of *Alternaria malorum* in this study

Location	The number of samples collected	The number of *Alternaria malorum* strains
Chaharmahal and Bakhtiari	2	4
Esfahan	2	3
Hamedan	7	16
Kerman	4	9
Kermanshah	5	8
Kohgiluyeh and Boyer-Ahmad	3	7
Kurdistan	4	9

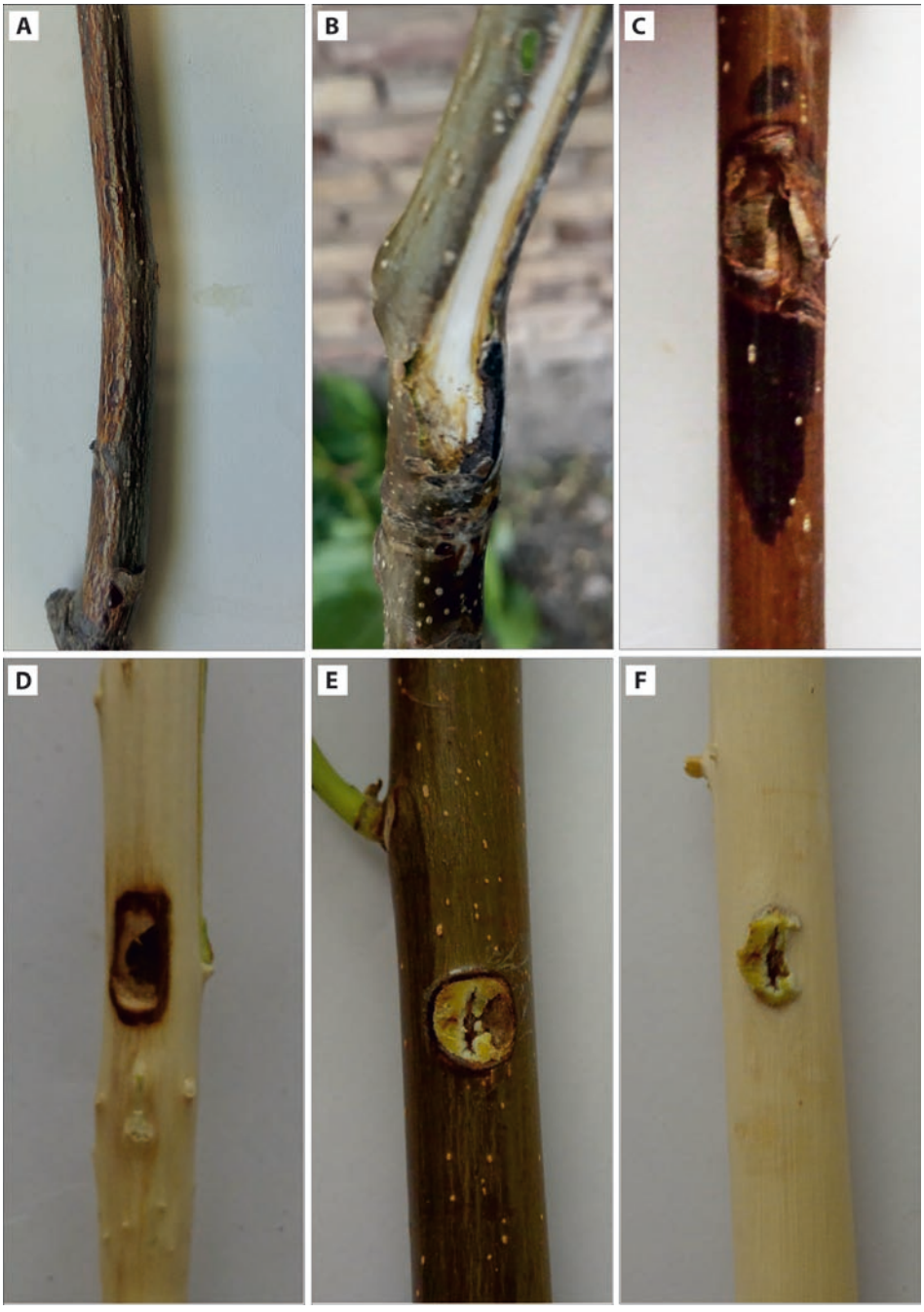

Fig. 1. A–B – natural symptoms of bark canker caused by *Alternaria malorum* on walnut trees; C–D – artificial symptoms after inoculation with *A. malorum* under laboratory conditions; E–F – control shoots without any symptoms

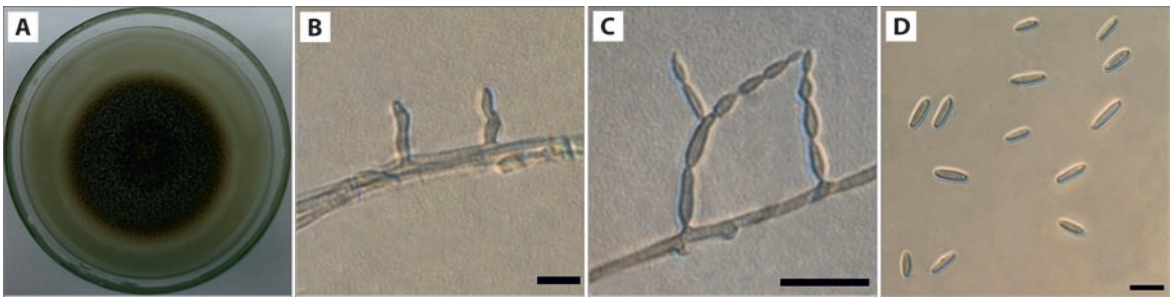

Fig. 2. *Alternaria malorum* SB32 after 7 days on potato carrot agar (PCA): A – surface of colony; B – conidiophores; C – conidial chain; D – conidia. Scale bars = 10 µm

5–12 µm × 3–4 µm in size (Figs. 2C–D). Morphological and cultural characteristics of purified isolates were in accordance with the description of *A. malorum* (Dugan *et al.* 1995; Braun *et al.* 2003; Goetz and Dugan 2006). Since the morphological features of all isolates were similar, one isolate (SB32) was selected as a representative for molecular analysis. To confirm preliminary identification based on morphological characteristics, the extraction protocol of Sharma *et al.* (2002) was used to extract genomic DNA from the representative isolate grown on a PCA plate. The internal transcribed spacer (ITS) region of the representative isolate was amplified using ITS1 and ITS4 primers (Hoff *et al.* 2004). The reaction mixture contained 10 ng of genomic DNA, 1 µM of each primer, 0.2 mM of dNTPs (CinnaGen, Iran), 1X PCR buffer, 2.5 mM $MgCl_2$ and 1 U Taq DNA polymerase (CinnaGen, Iran). PCR conditions for ITS included an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were purified and sequenced through Microsynth Company (The Swiss DNA Company, Bern, Switzerland). The sequence was adjusted using BioEdit v.7.0.5.2 where necessary (Hall 1999). A BLASTn search was used to compare the sequence generated in this study with those of the NCBI (National Center for Biotechnology Information) database. Homology search indicated the highest similarity (99.82–100% identity, query cover: 100%) with the ITS sequence of authentic strains of *A. malorum*. The novel generated sequence in this study was deposited in GenBank (MZ540328 Accession NO.). Data sequences included ITS sequence of isolate SB 32, sequences of *A. malorum* from other hosts and sequences of species closely related to *A. malorum* were aligned using ClustalW. A phylogenetic tree of the ITS sequences was constructed through maximum likelihood analysis with 1000 bootstrap replications in MEGA 7 (Kumar *et al.* 2016) with the Kimura-2-parameter nucleotide substitution model (Kimura 1980). The tree was rooted to *Dendryphiella triticicola* CBS 117147. Molecular assays by maximum likelihood analyses confirmed the phylogenetic position of isolate SB 32 among valid isolates of *A. malorum* (Fig. 3). Confirmation of pathogenicity was performed with seven representative isolates (one isolate from each province) in three replicates and the experiment was repeated twice. A total of 24 healthy shoots were inoculated after surface sterilization with 70% ethanol. Under-bark inoculation was performed using a sterile metal cork borer to wound the bark and to insert a mycelial plug of a 7-day-old fungal colony into the wound. Each wound was wrapped in Para-film to maintain moist conditions. Three healthy shoots were inoculated with sterile agar plugs as negative controls (Bagherabadi *et al.* 2017). Three weeks after inoculation, all inoculated shoots showed necrotic lesions similar to those observed on walnut trees while no symptoms were observed on control shoots (Figs. 1C–F). Once disease symptoms appeared, the fungus was re-isolated from the infected shoots on PDA and compared morphologically with the isolates obtained from natural symptoms. *Alternaria malorum* was consistently isolated from artificially inoculated shoots only. *Alternaria malorum* (Ruehle) U. Braun, Crous and Dugan spent most of its nomenclatural history as *Cladosporium malorum* Ruehle (Goetz and Dugan 2006). *Cladosporium malorum* (Ruehle) was transferred to *A. malorum* by Braun *et al.* (2003) based on differences in conidiogenesis and the structure of the conidiogenous loci, further supported by molecular data (Conidiogenesis is similar to that of the genus *Alternaria* and other species in the Pleosporaceae/-Pleosporales). Crous *et al.* (2009) recognized this species as *Chalastospora gossypii.* Woudenberg *et al.* (2013) suggested that *Ch. gossypii,* formerly known as *A. malorum*, belonged to section *Chalastospora* within genus *Alternaria*. *Alternaria malorum* was first reported by Ruehle (1931) as a pathogen on ripe apples and ripe cherries (Dugan *et al.* 1995). Goetz and Dugan (2006) reported this species as a fungal pathogen of cherry tomato and confirmed its appropriate classification in the genus *Alternaria*. In Iran, this species was first reported as *C. malorum on Hordeum vulgare* (Asgari *et al.* 2004).

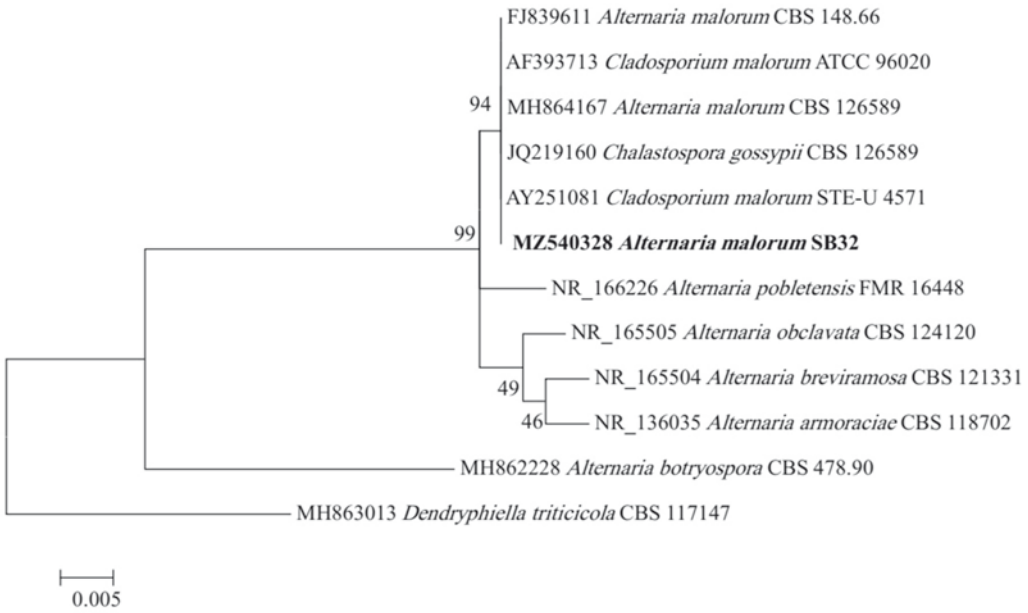

Fig. 3. Maximum likelihood phylogenetic tree based on ITS sequence of isolate SB32 and other ITS sequences of *Alternaria* spp. from GenBank. Numbers at the nodes are the bootstrap values obtained for 1,000 replicates. The tree is rooted to *Dendryphiella triticicola* CBS 117147

In a study conducted by Davari *et al.* (2011), *A. malorum* was reported from petroleum polluted soils. Hergholi *et al.* (2015) reported *A. malorum* as an endophyte of *Vitis vinifera*. It has been reported as a fungus associated with declined Persian oak trees (Alidadi *et al.* 2018). Molecular identification based on the ITS region supported the clear phylogenetic position of representative isolate SB 32 accurately. Furthermore, morphological distinguishing characteristics of isolates obtained in this study from closely related species confirmed the highest similarity of these isolates to *A. malorum*. The results of pathogenicity tests revealed that representative isolates of *A. malorum* were pathogenic on walnut shoots. In conclusion, since there have been no previous reports of *A. malorum* causing bark canker on walnut, we believe that this is the first report of *A. malorum* as a new fungal agent of bark canker on walnut trees. Our research included examination of its morphological characteristics and molecular identification, as well as testing its pathogenicity to fulfill Koch's postulates.

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