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Colletotrichum karstii, causal agent of anthracnose on ornamental *Ficus benjamina* in Iran

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Abstract

Anthracnose, a leaf disease caused by *Colletotrichum* species, is a major threat to ornamental plants worldwide. This study aimed to identify the causal agent of anthracnose affecting *Ficus benjamina* plants in greenhouses around Amol, Mazandaran Province, northern Iran. Fungal isolation from infected leaves revealed a *Colletotrichum* species, which was confirmed as *C. karstii* through morphological, cultural characteristics, and partial β-tubulin gene sequencing. Pathogenicity tests fulfilled Koch's postulates by reproducing anthracnose symptoms on inoculated *F. benjamina* plants. This study establishes *C. karstii* as the first reported causal agent of anthracnose on *F. benjamina* in Iran, contributing to a better understanding of *C. karstii* impact on Iranian ornamental plants.

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Introduction

The fungal genus *Colletotrichum* encompasses a diverse array of plant pathogens responsible for a wide spectrum of diseases, including anthracnose, on a vast number of plant species (Hyde et al., 2009a; Liu et al., 2022). Due to its substantial economic impact, the genus *Colletotrichum* ranks among the top ten fungal pathogens worldwide (Dean et al., 2012). Recent estimates suggest that this genus infects over 2200 plant species (Farr & Rossman, 2023). Accurate identification and characterization of plant pathogens are crucial for effective disease management strategies (Cai et al., 2009; Alizadeh et al., 2015). This is particularly important for pathogens that infect crops, ornamental plants, and other economically valuable plants (Nourmohammadi et al., 2023). By understanding the diversity, biology, and ecology of these pathogens, researchers and practitioners can develop targeted control measures that minimize crop losses and economic impacts (Alizadeh et al., 2022). *Colletotrichum* is a genus of particular concern due to its wide host range and ability to cause severe diseases on a variety of plants (Hyde et al., 2009). The genus is responsible for anthracnose, a disease that can affect fruits, vegetables, cereals, legumes, and ornamental plants (Cannon et al., 2012). *Colletotrichum* species have been implicated in significant crop losses worldwide, causing substantial economic damage to agricultural industries (Dean et al., 2012). Traditionally, the identification of *Colletotrichum* species relied heavily on morphological and cultural characteristics (Sutton, 1980). However, these methods have proven to be insufficient for accurate species delineation due to the lack of distinct and reliable morphological characters within the genus (Hyde et al., 2009b). The development of a more robust and reliable classification system for *Colletotrichum* has been a significant challenge for researchers. The advent of molecular techniques has revolutionized the field of *Colletotrichum* systematics (Cannon et al., 2012). DNA sequence data analysis, particularly through

phylogenetic analysis, has provided a powerful tool for differentiating species within large and complex groupings within the genus. These analyses have resulted in the identification of numerous novel species previously obscured within established species complexes (Prihastuti et al., 2009; Damm et al., 2012a, 2012b, 2014, 2019; Weir et al., 2012; Liu et al., 2015; Tao et al., 2013; Crouch, 2014; Marin-Felix et al., 2017; Liu et al., 2022; Alizadeh et al., 2022). Iran is home to a diverse range of plant species, providing a suitable habitat for various *Colletotrichum* species (Noroozi et al., 20016). In spite of recent studies (Alizadeh et al., 2015; Akbarzadeh et al., 2023; Alizadeh et al., 2022; Nourmohammadi et al., 2023) comprehensive knowledge regarding the diversity and distribution of *Colletotrichum* species in Iran still remains limited. Understanding the *Colletotrichum* species present in Iran is essential for developing effective disease management strategies for Iranian agriculture and horticulture (Alizadeh et al., 2015).

The present study aims to diagnose the causal agent of anthracnose symptoms on *Ficus benjamina* L. (*Moraceae*) in many greenhouses in Amol province, Iran using phylogeny combined with morphology. The findings of this study will contribute to a better understanding of *Colletotrichum* diversity in Iran and its potential impact on economically important plants. This information is crucial for developing effective disease management strategies for these plants, minimizing crop losses and economic impacts on Iran's agriculture.

Material and Methods

Isolation of the fungal pathogen

This study investigated an anthracnose disease outbreak affecting *F. benjamina* plants in greenhouses located near Amol, northern Iran (September 2013). To isolate the causal agent, leaf discs (approximately 1 cm in diameter) were excised from symptomatic leaves exhibiting characteristic lesions. The discs underwent the surface sterilization procedure including washing with tap water, disinfection in a 2% sodium hypochlorite solution for 1 minute, and three rinses with sterile distilled water. Following sterilization, the leaf discs were placed on moistened paper towels within sterile Petri dishes. These dishes were incubated in darkness at a temperature range of 20–25°C for one week.

Fungal culture and morphological characterization

After a week of incubation, fungal growth indicative of *Colletotrichum* spp. (saffron-yellow conidial masses) was observed on the leaf discs. Single spore isolation techniques were employed to obtain a pure culture (Goh, 1999), which was subsequently deposited in the Mycology Laboratory of the Faculty of Agriculture, Azarbaijan Shahid Madani University, Tabriz, Iran (Accession number: AZFC 246).

Morphological characteristics of the fungal isolate were assessed using oatmeal agar (OMA, Crous et al., 2009) medium following (Alizadeh et al., 2015). Colony appearance, color, and the presence of conidial structures were documented. Microscopic examination focused on conidiomata, conidia, and perithecia (sexual fruiting bodies) formed on synthetic nutrient agar (SNA, Nirenberg, 1976) plates (having 0.5 cm slices of *Anthriscus sylvestris* (L.) Hoffm. stems for better sporulation) after an incubation period of 3-4 weeks. Measurements of various fungal structures (conidia and ascospores) were recorded (Damm et al., 2007).

Molecular identification

To confirm the initial morphological identification, a molecular approach was employed. Polymerase Chain Reaction (PCR) was used to amplify a partial fragment of the β-tubulin (*TUB2*) gene. Specific primers, T1 (O'Donnell & Cigelnik, 1997) and Bt-2b (Glass & Donaldson, 1995), were used for this purpose. Polymerase Chain Reaction (PCR) was performed following the protocol described by (Nourmohammadi et al., 2023). The amplified DNA fragments were then sequenced using the Sanger sequencing method by a commercial service provider (Biomagic Gene Company, BMG, China) using the same primers that were used for PCR. Geneious software version 5.6 was used to view, edit, and assemble the obtained DNA sequence. To compare this sequence with previously published sequences, particularly those from type and reference strains, the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) from GenBank (http://ncbi.nlm.nih.gov/genbank) was employed. Sequences with significant similarity were then included in the multiple sequence alignment as reference strains (Fig. 1). Additionally, *Monilochaetes infuscans* Halst. ex Harter strain CBS 869.96 was chosen as the outgroup taxon for the analysis (Fig. 1).

The MAFFT online server (Katoh & Stadley, 2013) was used to perform the multiple sequence alignment. The Q-INS-i algorithm (Katoh & Stadley, 2013) which is the latest available version, was employed during this alignment process. Maximum likelihood phylogenetic analysis will be conducted using RAxML software

(Stamatakis, 2014) with the GTRGAMMA model and 1000 bootstrap replicates. This analysis will be performed on the TrEase web server (http://thineslab.senckenberg.de/trease/) using the latest available version. The sequence generated in this study deposited in GenBank, and the accession number provided.

Pathogenicity test

Koch's postulates were fulfilled to confirm the pathogenicity of the isolated *Colletotrichum karstii* You L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai strain (AZFC 246) on *F. benjamina*. A conidial suspension (10^6 spores/ml) was prepared from a seven-day-old culture. This suspension was then sprayed onto leaves of healthy *F. benjamina* plants (approximately 40-50 cm tall). The inoculated plants were covered with plastic bags to maintain humidity and placed in a greenhouse at a controlled temperature of 25°C ± 5°C. After four days, the inoculated plants were evaluated for the development of anthracnose symptoms resembling those observed in the original outbreak. Additionally, control plants were maintained without inoculation. Re-isolation of the fungus from symptomatic leaves on the inoculated plants fulfilled the final step of Koch's postulates.

Results

In the *TUB2* phylogenetic analysis of the *Colletotrichum boninense* Moriwaki, Toy. Sato & Tsukib. Complex, 53 isolates were examined along with the outgroup and 514 characters, including alignment gaps were processed. Of these characters, 154 were parsimony-informative, 229 were variable, and 285 were constant. In Maximum Likelihood tree the examined isolate AZFC 246 (GenBank accession number: MG490986) from *F. benjamina* placed in a well-supported clade (bootstrap: 94) with the type strain of *C. karstii* Y.L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai (CGMCC3.14194) (Fig. 1). A BLAST search of the resulting sequence showed 100 % similarity to the ex-type strain of *C. karstii*, CGMCC 3.14194 (HM585428; Yang et al., 2011).

Taxonomy

Molecular and morphological analyses identified the isolates from *F. benjamina* as *C. karstii*, a species within the *C. boninense* complex. A detailed morphological characterization of the representative isolate used in this study is provided below.

Colletotrichum karstii Y.L. Yang, Z.Y. Liu, K.D. Hyde & L. Cai, Cryptogamie Mycologie 32: 241 (2011) (Fig. 2).

On *Anthriscus* stems. The fungus formed clusters of conidiomata that were flask-shaped and colorless to light brown. These structures housed conidiophores that produced single-celled, elongated conidia. These spores had smooth walls, were colorless, and measured around 10-15 µm long and 4-6 µm wide, mean = 14 × 5.0 µm, L/W ratio = 2.75.

On SNA. Further analysis on SNA media revealed the development of sexual fruiting bodies (ascomata) after several weeks. These ascomata (perithecia) emerged as individual structures (solitary) after 3-4 weeks. They appeared either on the surface (superficial) or embedded within (immersed) the agar medium. The ascomata lacked a compact tissue base (non-stromatic) and were round to pear-shaped (globose to obpyriform). Each possessed an opening (ostiole) for spore release, had a smooth, hairless (glabrous) surface, and were brown in color. Their size ranged from 98–125 µm in diameter and 92–114 µm in height. The ascomata contained single-walled (unitunicate) spore sacs (asci), each housing eight spores (8-spored). These asci came in various shapes, ranging from cylindrical to club-shaped (clavate or fusiform). They tapered towards both ends (apex and base) and had smooth walls. Their size ranged from 40–53 µm in length and 10–12 µm in width. The spores (ascospores) were initially unsegmented (aseptate) but could develop internal divisions (septate) as they matured. They were colorless (hyaline) and had smooth walls. Their shape varied from spindle-shaped (fusiform) to egg-shaped (ovoid), with a slight curve. The size range was (15–)16.75–17.25(–18) µm in length and (5–)5.5–6.5(–7.5) µm in width, mean = 17 × 6 µm, L/W ratio = 2.75.

Analysis revealed the absence of thick-walled resting spores (chlamydospores) and asexual fruiting structures (conidiomata). Instead, spore-producing structures (conidiophores) arose directly from the vegetative hyphae (fungal threads). Furthermore, hair-like structures (setae) were not observed (Fig. 2).

Cultural characteristics

On SNA media, the fungal colonies appear flat with smooth margins. Initially, they are colorless and cover both the filter paper and *Anthriscus* stem fragment with orange clusters of asexual spores. The unseen fungal body beneath the surface (reverse) remains colorless with scattered gray patches, particularly under the filter

paper. These colonies reach a diameter of 5–6 cm within a week and expand to 7-8 cm after ten days.

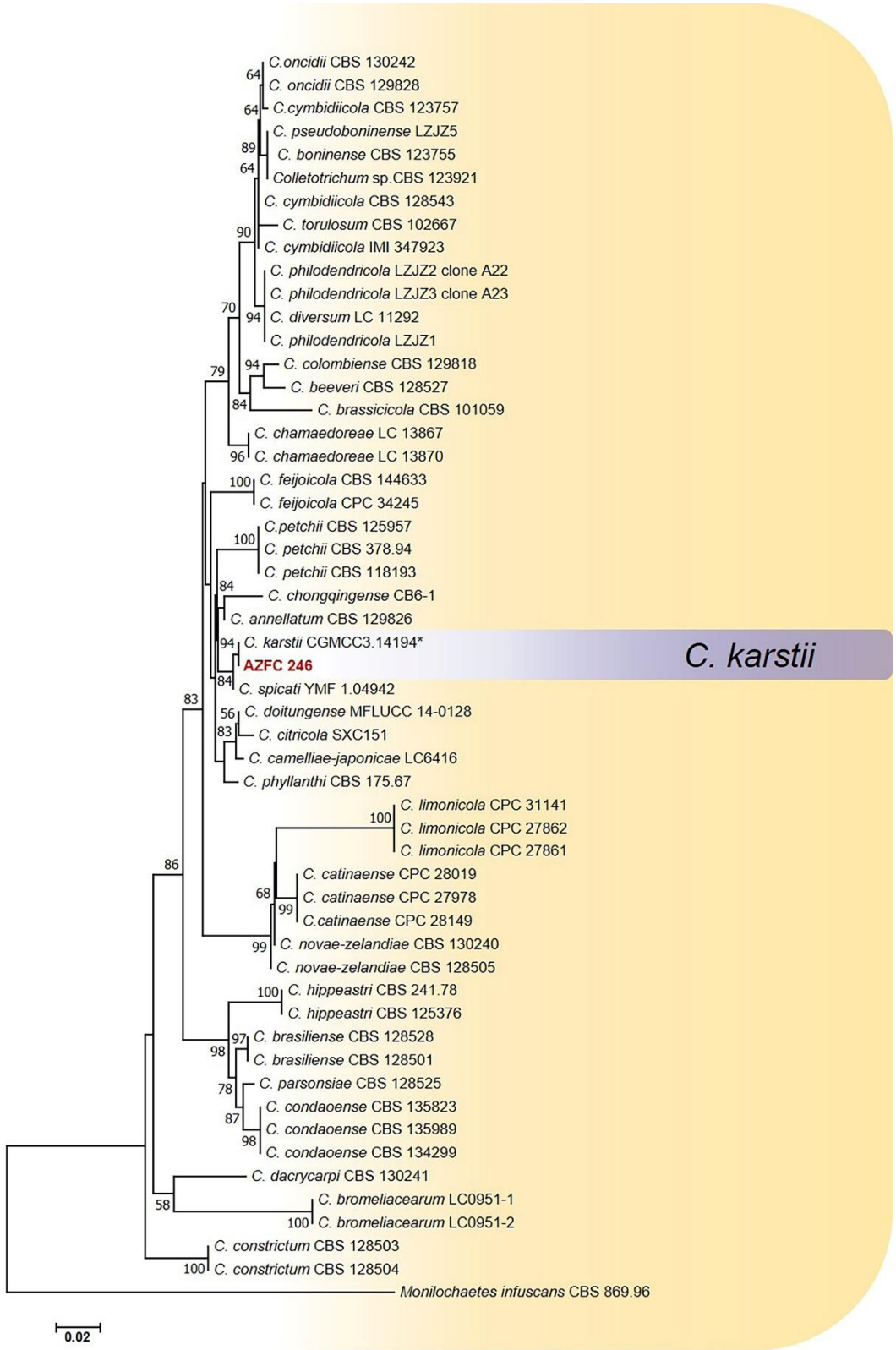

Fig. 1. Phylogram generated from Maximum Likelihood analysis of the *TUB2* sequence alignment of the Boninense complex. Bootstrap values above 50 are shown at the nodes. *Monilochaetes infuscans* strain CBS 869.96 is used as outgroup. The newly generated isolate in this study is in red.

On OA media, the colonies exhibit similar flat growth with smooth margins. Their color varies from light brown or buff to slightly rosy or pale salmon. The center of the colonies is densely packed with orange to gray clusters of asexual spores. The unseen fungal body beneath the surface displays a range of colors, including brown, light brown, buff, rosy, and honey. These colonies reach a diameter of 5 cm within a week and expand to 7 cm after ten days.

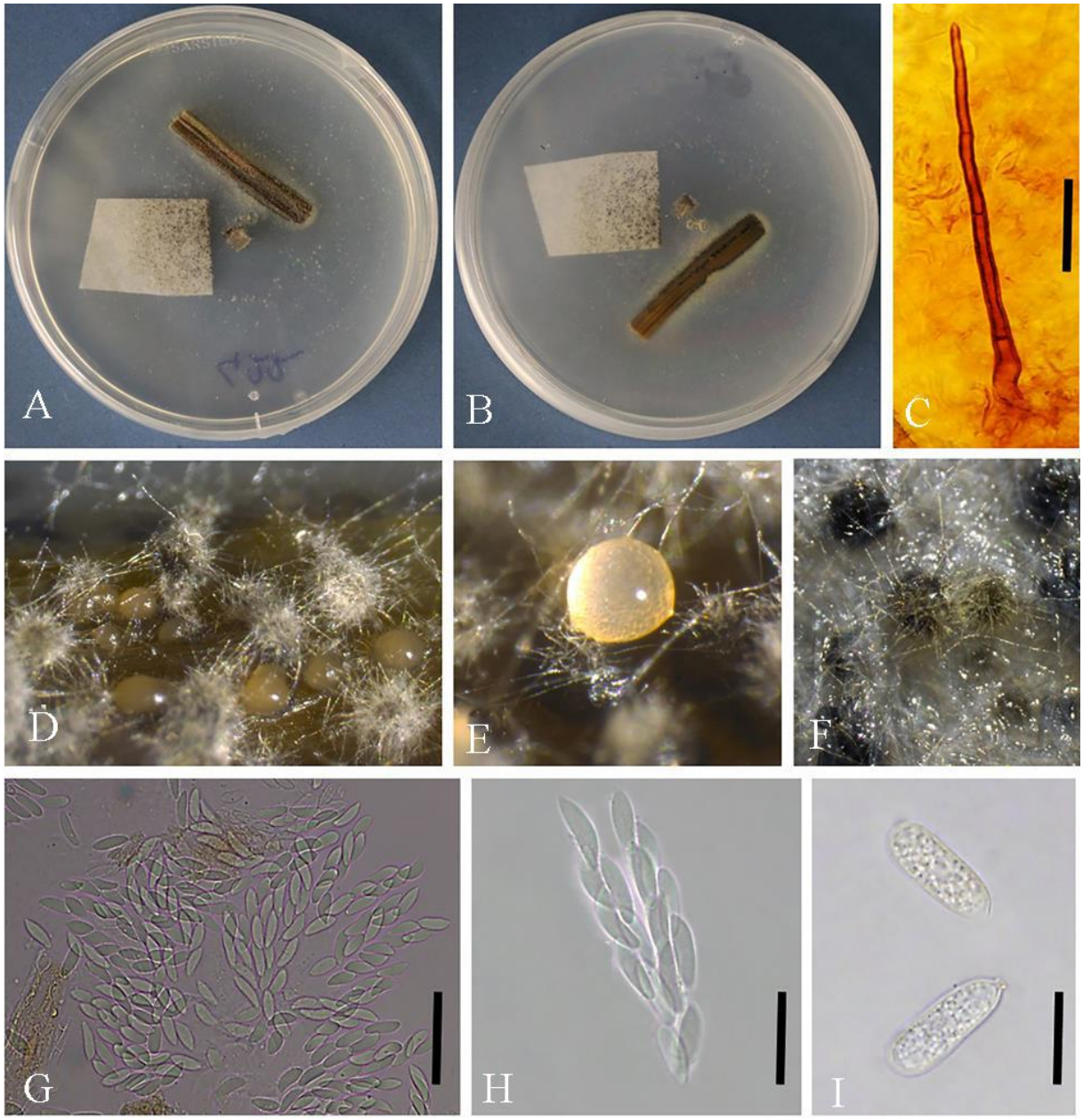

Fig. 2. *Colletotrichum karstii* (AZFC 245). (A, B) Colony on SNA after 14 days at 25 °C, a. upper and b. reverse side. (C) Setae. (D-E) Ascomata and Conidial mass on *Anthriscus sylvestris* stem. (F) Ascomata on filter paper. (G,-H). Asci and ascospores.(I) Conidia. Scale bars: c, h = 20 µm, g = 50 µm, i = 10 µm.

Fulfillment of Koch's Postulates

Inoculation of *F. benjamina* plants with a conidial suspension of AZFC 246 isolate resulted in the development of characteristic anthracnose symptoms after four days, replicating the disease observed in the greenhouses (Fig. 3). Conversely, control plants remained symptomless. Furthermore, the re-isolation of the fungus from the symptomatic leaves confirms Koch's postulates, solidifying the role of AZFC 246 isolate as the causative agent of the observed *F. benjamina* anthracnose disease isolates.

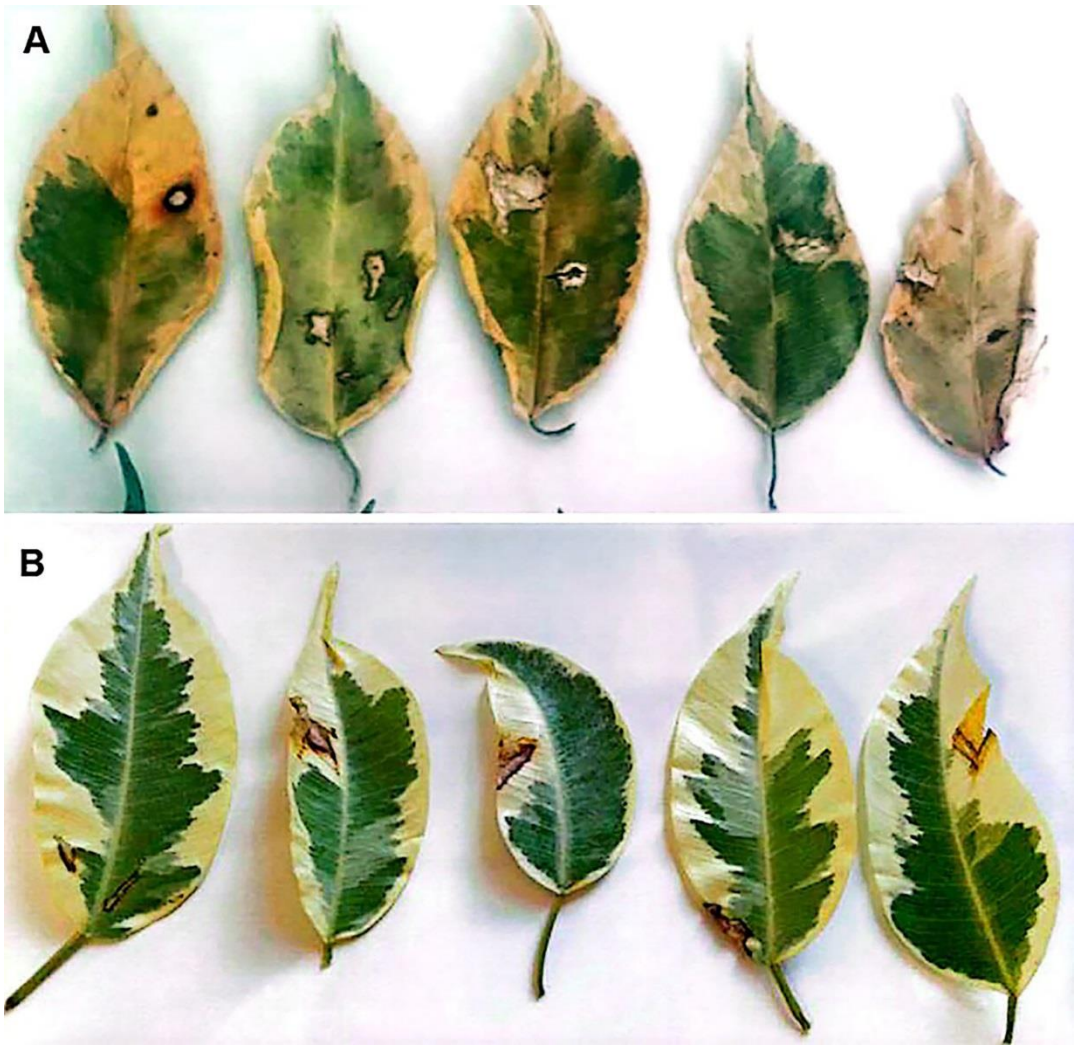

Fig. 3. Anthracnose symptoms on leaves of *Ficus benjamina*: (A) Naturally infected leaves. (B) Leaves of inoculated plants by *Colletotrichum karstii* strain AZFC 246.

Discussion

This study identified and characterized the causal agent of anthracnose disease affecting *F. benjamina* plant in Iranian greenhouses as *C. karstii*. This finding not only represents the first report of *C. karstii* causing anthracnose on *F. benjamina* in Iran but also expands our understanding of the fungus' host range and potential threat to the country's ornamental plants. Confirming *C. karstii* relied on a combined approach of morphological analysis and partial β-tubulin gene sequencing. This highlights the limitations of morphology alone (Hyde et al., 2009a, b), particularly given the inherent genetic and morphological variability documented for *C. karstii* (Damm et al., 2012b). This variability can manifest in diverse ways, including significant differences in asexual spore size and structure (*e.g.*, scattered clusters vs. dense masses) (Damm et al., 2012b). *Colletotrichum karstii*, first described by Yang et al. (2011), exhibits a dualistic lifestyle, acting as both a pathogen and an endophyte (internal symbiont) in various plant species (Yang et al., 2011). This finding, along with its documented presence as an endophyte in Iran (Alizadeh et al., 2015), warrants further investigations into its potential impact on Iranian plant health. *Colletotrichum karstii* is a significant plant pathogen with a broad host range. It infects a wide range of species, including orchids (*Bletilla ochracea* Schltr.) (Tao et al., 2013) and passion fruit (*Passiflora edulis* Sims) (Pileggi et al., 2009). This study adds *F. benjamina* to the growing list of susceptible hosts and highlights the potential threat *C. karstii* poses to Iranian ornamental plants. *Colletotrichum karstii* is recognized as one of the most geographically widespread members of the *C. boninense* complex (Damm et al., 2012b). While previously reported as part of Iran's Funga (Alizadeh et al., 2015), this study provides the first evidence of its role in causing *F. benjamina* anthracnose within the country. Further investigations are necessary to determine its complete geographical distribution and prevalence in Iran, particularly on other ornamentals or

economically important crops to elucidating the complete spectrum of susceptible hosts in Iran, especially economically important crops and ornamentals, exploring the geographical distribution and prevalence of *C. karstii* in Iran and developing effective disease management strategies for *C. karstii*-induced anthracnose on *F. benjamina* in greenhouses. This may involve exploring fungicide options, cultural practices, or the use of resistant plant varieties.

By addressing these knowledge gaps and pursuing these future research avenues, this study contributes valuable information to our understanding of *C. karstii* and its role in plant diseases within Iran.

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Conflict of interest

The authors declare that there are no conflicts of interest present.

CRediT author statement

E. Abdinejhad: Laboratory works & writing original draft. **A. Alizadeh**: Supervision, methodology, sampling, writing, reviewing & editing. **A. Shirzad**: counseling, reviewing & editing.

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