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Biscogniauxia rosacearum as a pathogen associated with pistachio trees in Fars Province, Iran

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Abstract

Pistachio (*Pistacia vera* L.), a member of the *Anacardiaceae* family, is one of the most worthwhile crops worldwide. Iran has the most favorable climate for cultivating pistachio trees. Charcoal canker disease has spread in Iran due to environmental climate changes such as drought and abnormally high temperatures. During the summer of 2023, infected pistachio trees in Fars Province died and displayed charcoal canker symptoms with carbonaceous stromata. In total, five isolates of a fungal species were obtained from necrotic wood tissues of declining pistachio trees in Sarvestan County, Fars Province. Morphological studies showed that none of the fungal isolates being examined were able to produce conidia or ascospores on different media, even after changing conditions such as pH, temperature, or light, making it impossible to perform morphological analysis of pycnidial and perithecial structures. Therefore, molecular identification was performed by phylogenetic analysis of the ITS-rDNA region and the β-tubulin (*tub2*) gene. The results revealed that the isolates formed a monophyletic group with *Biscogniauxia rosacearum* in a well-supported clade (posterior probability = 100). Pathogenicity tests showed that the isolates were pathogenic on detached pistachio branches, causing necrotic wood lesions on the bark that extended into the wood 30 days after inoculation. To the best of our knowledge, this study represents the first report of *B. rosacearum* associated with pistachio trees.

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Introduction

The pistachio nut (*Pistacia vera* L.) belongs to the family *Anacardiaceae* and is one of the most valuable agricultural crops worldwide (Tomaino et al., 2010). In Iran, the desert climate provides suitable conditions for pistachio growth (Roostaei, 2004). Therefore, many regions including Kerman, Semnan, Damghan, Yazd, and Fars Provinces are suitable for pistachio cultivation (Esfandiyari et al., 2012). One of the major factors limiting pistachio production is the presence of soil-borne plant pathogenic fungi and fungus-like agents. The genera *Pythium*, *Phytophthora*, *Verticillium*, *Armillaria*, and *Rosellinia* are important pathogens of pistachio trees with worldwide distribution (Alaei et al., 2017; Aydın & Ünal, 2021). The genus *Biscogniauxia* Kuntze, which belongs to the family *Xylariaceae*, has a wide host range and includes 52 reported species that are known to be responsible for the decline in dicotyledonous angiosperm trees (Vasilyeva & Stephenson, 2010; Vasilyeva et al., 2012). This genus has been reported worldwide, including in the United States, Africa, Europe, and Asia (Whalley et al., 2012). This fungus is considered as an opportunistic pathogen that attacks weakened trees under climate-related stresses such as abnormally high temperatures and drought. It is believed to live as an endophytic fungus within the internal tissues of living plants, surviving in a latent phase in healthy trees for their entire lifetime or for an extended period without causing visible symptoms (Linaldeddu et al., 2011; Vannini et al., 2009; Rodriguez et al., 2008). *Biscogniauxia mediterranea* (de Not.) Kuntze is known as one of the causal agents of charcoal cankers in the inner bark of hardwood species and has been reported on various tree hosts worldwide (Linaldeddu et al., 2011; Ahmadi et al., 2022). In the

past two decades, oak charcoal canker disease has spread in the Zagros forests of Iran due to drought stress (Mirabolfathy et al., 2013; Mansouri Daneshvar et al., 2019). During the summer of 2023, infected pistachio trees in the Fars Province died and exhibited charcoal canker symptoms, including carbonaceous stromata. Therefore, the main objective of the present study was to identify and characterize isolates of *Biscogniauxia* collected from pistachio trees in the Fars Province based on morphological characteristics and molecular analysis using phylogenetic inference of DNA sequence data from the β-tubulin (*tub2*) and ITS-rDNA regions.

Materials and Methods

Sampling and fungal isolation

From early September to late March 2023, surveys were conducted to identify charcoal canker disease in pistachio orchards in the Sarvestan region of Fars Province. Samples of carbonaceous stromata from the trunks and branches of pistachio trees were collected, placed in polyethylene bags, and transported to the laboratory. The samples were stored in a refrigerator at 4° C until fungal isolation was performed. To isolate the causal agent of pistachio charcoal canker disease, infected tissue samples were cut into 2–5 mm segments using a sterile scalpel, surface-disinfected in 1% sodium hypochlorite (NaClO) for 45 s., rinsed with sterile deionized water, dried on sterile paper towels, and cultured on potato dextrose agar (PDA) medium. The PDA medium consisted of 12 g/L potato extract, 10 g/L dextrose, and 1.2% agar, supplemented with 50 ppm Kanamycin to prevent bacterial contamination. The plates were incubated at 27°C and monitored daily for fungal growth. Pure cultures were obtained by the hyphal tip technique (Ahmadi et al., 2022).

Morphological identification

To identify the fungal species, all collected fungal isolates were morphologically evaluated and classified according to genus and species descriptions provided in valid taxonomic references (Ju & Rogers 2001; Mirabolfathy et al., 2013).

DNA extraction and sequencing

Fungal isolates were cultured on potato dextrose agar (PDA) at 25°C in the dark for five days. Subsequently, the mycelial plugs were transferred to 50 mL of potato dextrose broth (PDB; 10 g/L potato extract, 10 g/L dextrose) and incubated at 20°C on a rotary shaker at 120 rpm for seven days. The resulting mycelial biomass was harvested, flash-frozen in liquid nitrogen, and ground into fine powder. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method described by Murray & Thompson (1980).The DNA concentrations were adjusted to 25 ng/µL and stored at −20°C until further analysis. The pair of primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') andITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990), and T1 (5'-AACATGCGTGAGATTGTAAGT-3') (O'Donnell and Cigelnik, 1997)/Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') (Glass & Donaldson, 1995) were used to amplify ITS-rDNA regions and β-tubulin gene, respectively. The PCR reaction mixtures were prepared in a final volume of 20 µl, comprising 0.04 µM of each primer (Microsynth, Switzerland), 0.4 µM dNTPs (MBI Fermentas, Germany), 1× Dream Taq Buffer (MBI Fermentas), and 0.4 U Dream Taq DNA polymerase (MBI Fermentas). The cycling conditions consisted of an initial denaturation at 96°C for 6 min, followed by 35 cycles of 96°C for 30 s, annealing at 52 °C for ITS-rDNA at 58 °C for β-tubulin at 45 s, and extension at 72°C for 90 s. The final extension step was performed at 72°C for 8 min (Ahmadi et al., 2022). Sequencing was performed in both directions using the DNA Sequencing Service of Macrogen (Macrogen Inc., South Korea). DNA sequences were checked and manually edited using Geneious software (Biomatters Inc., USA).

Phylogenetic analysis

The ITS-rDNA and β-tubulin sequences were blasted separately using Megablast to identify their closest neighbors. Subsequently, all ITS-rDNA and partial β-tubulin sequences were concatenated and used for phylogenetic analysis to determine the taxonomic placement of the *Biscogniauxia* isolates (Table 1). A phylogenetic tree was constructed based on concatenated sequences of β-tubulin (1920 characters) and ITS-rDNA (882 characters). The final alignments were performed using Geneious version 7 (Biomatters, USA). The combined alignment consisted of 2685 characters. The best evolutionary model was determined using the ModelTest option from MEGA 11. Phylogenetic trees were constructed using the maximum likelihood algorithm (Tamura *et al*., 2013). *Hypoxylon rubiginosum* (BCRC34116) was used as the outgroup taxon to root the phylogenetic tree because of its close taxonomic relationship with the *Biscogniauxia* species

(Mirabolfathy et al., 2011).

Pathogenicity test on detached branches

Pathogenicity tests were conducted on the detached pistachio branches. Healthy, actively growing branches were collected and trimmed to remove leaves and lateral shoots. The branches were cut into segments of 20–22 cm long and 1–2 cm in diameter, washed, and the bark surfaces were disinfected by spraying with 70% ethanol. Both ends of the segments were sealed with warm melted paraffin wax to reduce dehydration during incubation. Three T-shaped cuts were made on the bark of each segment using a sterile scalpel. A six mm PDA plug taken from the margin of a 5-day-old colony of each isolate was inserted into each cut. The bark was then replaced, and the inoculation site was wrapped with Parafilm. Non-inoculated PDA plugs were used as controls. All inoculated branches were placed in humid chambers, which were plastic containers kept at 100% relative humidity by adding 250–300 mL of water and stored at room temperature (Ahmadi et al., 2022).

Table 1. *Biscogniauxia* isolates used in phylogenetic analyses in this study.

Fungal species	Isolate	Collecting data	GenBank accession no.	
			ITS-rDNA	β-tubulin (*tub2*)
Biscogniauxia anceps	YMJ 123	Rogers et al., 1996	EF026132	AY951671
B. arima	YMJ 122	Ju et al., 1998	EF026150	AY951672
B. atropunctata	YMJ 128	Ju et al., 1998	JX507799	AY951673
B. capnodes	YMJ 138	Ju et al., 1998	EF026131	AY951675
B. cylindrispora	YMJ 89092701	Ju & Rogers, 2001	EF026133	AY951679
B. formosana	YMJ 89032201	Ju & Rogers, 2001	JX507802	AY951680
B. granmo	YMJ 135	Ju et al.,1998	JX507803	AY951681
B. latirima	YMJ 89101101	Ju & Rogers, 2001	JX507804	AY951682
B. latirima	YMJ 90080703	Hsieh et al., 2005	EF026135	AY951683
B. mediterranea	YMJ 147	Ju et al., 1998	EF026134	AY951684
B. nummularia	H86	Pazoutova et al., 2012	GQ428318	GQ428324
B. simplicior	YMJ 136	Hsieh et al., 2005	EF026130	AY951686
B. uniapiculata	YMJ 90080608	Hsieh et al., 2005	JX507805	AY951687
B. philippinensis	YMJ 89041101	Ju & Rogers, 2001	EF026136	AY951685
B. rosacearum	IRNBS68	Sohrabi et al.2022	MW452324	MW456675
B. rosacearum	Bx26	Sohrabi et al.2022	KT253493	KT253527
B. rosacearum	**Pis-Iran1**	This study	PV489351	PV504632
B. rosacearum	**Pis-Iran2**	This study	PV489352	PV504633
B. rosacearum	**Pis-Iran3**	This study	PV489353	PV504634
B. rosacearum	**Pis-Iran4**	This study	PV489354	PV504635
B. rosacearum	**Pis-Iran5**	This study	PV489355	PV504636
Hypoxylon rubiginosum	YMJ 24	Ju & Rogers, 2001	EF026143	AY951751

Results

Morphological identification

Symptoms of pistachio charcoal canker included wilting, production of carbonaceous stromata on the trunks and branches of pistachio trees, and dieback, which ultimately led to tree mortality (Fig. 1a–b). In

total, five isolates were obtained from necrotic wood tissues of declining pistachio trees in the Sarvestan region of the Fars Province, with only one isolate collected from each infected tree. These five isolates were successfully recovered in October and November.

In general, the cultures showed white to gray colonies with aerial mycelium on PDA medium, while the reverse side of the colonies was dark brown to black (Fig. 1c–d). None of the five isolates produced conidia or ascospores on the tested media, making morphological analysis of pycnidial and perithecial structures impossible. The maximum, minimum, and optimum temperatures for growth on PDA were 42.5 °C, 10 °C, and 27 °C, respectively.

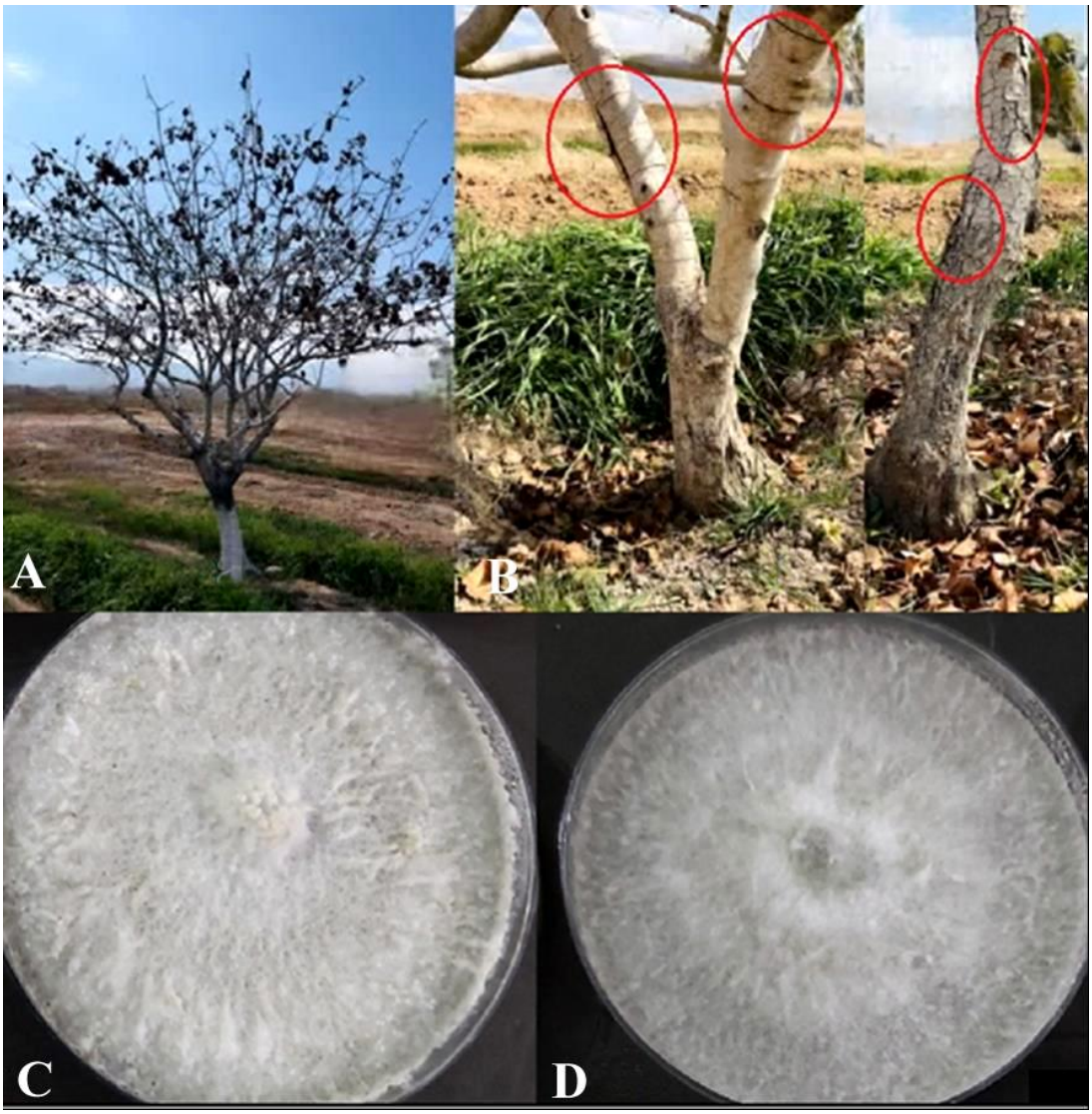

Fig. 1. *Biscogniauxia rosacearum*: Symptoms of charcoal canker on pistachio trees: (A) wilting, (B) Production of carbonaceous stromata on trunks and branches of pistachio trees showing dieback and death symptoms, (C and D) White to gray colonies with aerial mycelium on PDA after seven days of incubation.

Phylogenetic analysis

Multilocus phylogenetic analysis was performed using a two-gene phylogeny (*tub2* and ITS-rDNA) of *Biscogniauxia* species. PCR amplification and sequencing were successful for all the isolates. The obtained sequences were submitted to NCBI's GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers PV489351-PV489355 for ITS-rDNA and PV504632-PV504636 for *tub2* (Table 1). The

topology and branch lengths of the phylogenetic inferences are shown in Fig. 2, which distinguish the phylogenetic positions of isolates Pis-Iran1 to Pis-Iran 6 based on the combined dataset of βtub and ITS-rDNA sequences. The multigene phylogenies reveal that isolates Pis-Iran1 to Pis-Iran6 formed a monophyletic group with *B. rosacearum* (strain: BCRC34037) in a well-supported clade (posterior probability=100).

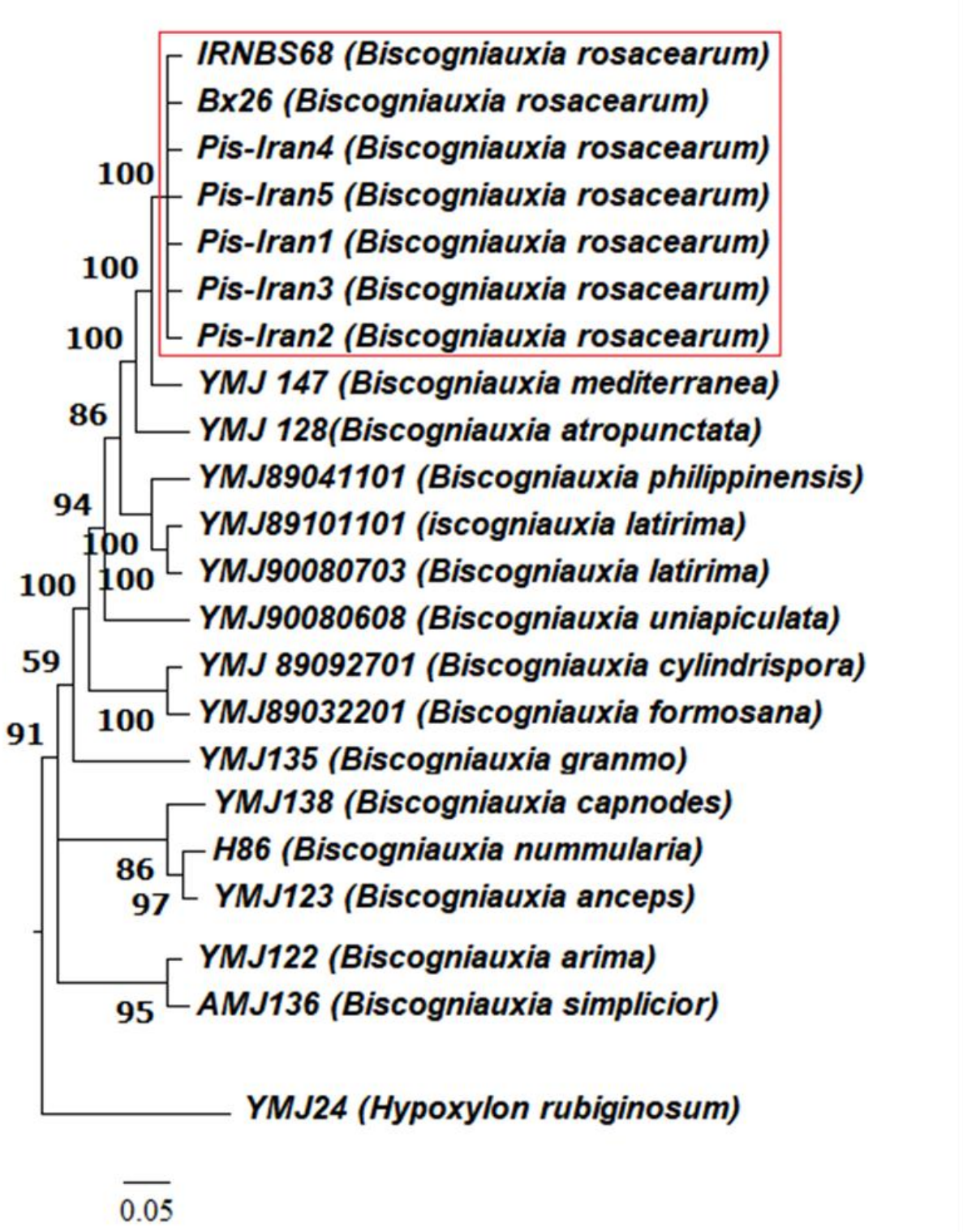

Fig. 2. Maximum likelihood phylogenetic tree based on the concatenated dataset of β-tubulin (*tub2*) and ITS-rDNA sequences of *Biscogniauxia* species. *Hypoxylon rubiginosum* was used as the outgroup. Iranian isolates from the present study are highlighted within a red box. Bootstrap values greater than 50% are indicated as percentages based on 1000 replicates. The scale bar represents 0.05 substitutions per site.

Pathogenicity test

Five isolates were pathogenic on detached pistachio branches, causing necrotic lesions on the bark that extended into the wood 30 d after inoculation. No lesions were observed on the non-inoculated control branches. Koch's postulates were confirmed by re-isolating the fungal isolates from infected tissues on PDA (Fig.3).

Fig. 3. Pathogenicity test of five *Biscogniauxia rosacearum* isolates on detached pistachio branches of Pistachio trees one month after inoculation: (A) Pis-Iran1, (B) Pis-Iran2, (C) Pis-Iran3, (D) Pis-Iran4, (E) Pis-Iran5, (F) non-inoculated control.

Discussion

Charcoal canker disease is recognized as an important problem in Iran,. Primarily, drought stress and subsequent changes in the water content of plant tissues can weaken the plant's defense systems and promote the development of fungal pathogens, such as *Biscogniauxia* species (Vannini et al., 2009; Corcobado et al., 2014; Ghanbary et al., 2017). In this study, the lack of diagnostic morphological characteristics in culture needed to identify the fungus at the species level prompted the use of molecular techniques to assess its identity. Thus, our diagnostic morphological characteristics was not consistent with those of previous studies (Raimondo et al., 2016; Rostamian et al., 2016; Bahmani et al., 2021; Sohrabi et al., 2022). Nevertheless, multilocus phylogenetic analysis of β-tubulin and ITS-rDNA sequences data indicated that *B rosacearum* is the causal agent of charcoal canker and a newly reported pathogen associated with pistachio trunk diseases in the Sarvestan region of Fars Province. These findings provide new insights into the protection of fruit orchards in this region.

Isolates were obtained only from the samples collected in October and November. In contrast, no isolates were recovered from carbonaceous stromata in late autumn and winter, as the infected tissues were completely rotten and easily disintegrated. Thus, our results are consistent with previous findings by Safaee et al. (2017) and Ahmadi et al. (2022) which showed that rotten tissues did not contain viable ascospores. Therefore,

sampling time is crucial for successful pathogen isolation.

Previous studies have demonstrated that *B. mediterranea* is one of the primary causal agents of charcoal canker affecting forest, fruit, and ornamental trees (Henriques et al., 2016; Raimondo et al., 2016). *Biscogniauxia rosacearum*, a newly described species within the genus *Biscogniauxia*, was initially reported on rosaceous hosts and has subsequently been identified as a pathogen on various other hosts, including *Quercus castaneifolia*, almond, and grapevine in Iran; *Platypus cylindrus* in Portugal; *Prunus domestica*, *Cydonia oblonga*, *Pyrus communis*, and *Quercus pubescens* in Italy, and *Quercus ilex*, *Holcus lanatus*, and *Pinus sylvestris* in Spain (Raimondo et al., 2016; Rostamian et al., 2016; Bahmani et al., 2021). To the best of our knowledge, this study represents the first report of *B. rosacearum* associated with pistachio trees both in Iran and globally. These findings suggest that broader and more extensive sampling efforts may be necessary to identify additional host species for this emerging pathogen.

The results of the pathogenicity test on pistachio branches demonstrated that all five isolates of *B. rosacearum* were pathogenic on pistachio. These findings are consistent with previous pathogenicity studies conducted on almond, pear, quince, and plum, which concluded that this fungus could act as a new pathogen in the hosts (Raimondo et al., 2016; Bahmani et al., 2021; Sohrabi et al., 2022).

In this study, *Phytophthora* species were detected exclusively in the crown regions of some pistachio trees, with no isolates obtained from the trunks. This finding is consistent with the widespread use of traditional flood irrigation in the surveyed orchards, which promotes the release and dispersal of zoospores and creates favorable conditions for the development of *Phytophthora*-induced root and crown rot diseases. Moreover, our results provide new evidence that pistachio trees can serve as woody hosts for *B. rosacearum*, a pathogen not previously reported on this host. This underscores the need for further research to better understand the pathogenic role and distribution of *B. rosacearum* in pistachio and other fruit trees across different regions of Iran. It is worth noting that although *Phytophthora* species were isolated from the collar region, this was not the primary focus of the study, as their presence on pistachio has already been well documented. Nonetheless, reporting their occurrence contributes to a more comprehensive understanding of the disease complex affecting pistachio trees.

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Conflict of interest

The authors have no conflicts of interest to declare.

CRediT author statement

F. Ghaderi: conceived and supervised the study, and was responsible for the overall manuscript preparation. **R. Rezaei**: assisted in data collection, data analysis, and manuscript writing.

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