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Short Notes

First report of *Biscogniauxia mediterranea* causing cankers on almond trees (*Prunus dulcis*)

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Summary. *Biscogniauxia mediterranea* is the causal agent of charcoal disease in *Quercus suber*, the main species of the dynamic ecosystem, known as "Montado", in the Alentejo region, Portugal. In the last years, almond orchards have been introduced in this region due to water availability through the Alqueva dam and the possibility of mechanical harvest. The high-density planting associated with mechanized harvesting and irrigation systems observed in these new orchards can potentiate the appearance of new diseases. In a survey conducted in March 2022, symptomatic diseased trees from Soleta and Vairo cultivars were detected in Beja, Portugal. From this material, we have isolated numerous cultures and could identify *B. mediterranea* from all individuals analyzed by molecular and morphological techniques. Pathogenicity tests were performed in almond plant material and successfully reisolated from lesions, confirming Koch's postulates. Phylogenetics analyses demonstrated the similarity between our sequences and sequences from *Quercus suber* worldwide. To our knowledge, this is the first report of *B. mediterranea* causing diseases on almond trees (*Prunus dulcis*) in Portugal and worldwide.

Keywords. *Biscogniauxia mediterranea*, *Prunus dulcis*, charcoal disease, pathogenicity, Koch's postulates.

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INTRODUCTION

Almond (*Prunus dulcis* Mill. D. A. Webb) is an important nut crop originating from southwest Asia and grown in other regions including the Mediterranean basin (Tomishina *et al.*, 2022). In Portugal, almond trees have been

traditionally cultivated in Douro/Trás-os-Montes and Algarve regions (respectively, north and south Portugal). Almond production in Portugal has recently increased, with introduction of commercial cultivars adapted for an irrigated system in new regions, such as Alentejo and Beira Interior (Faustino *et al.*, 2022). The Alentejo region is known for its agro-forestry-pastoral "Montado" ecosystem, characterized by presence of *Quercus* species such as *Q. suber* and *Q. ilex*. One of the most frequent diseases affecting *Quercus* trees in this region is charcoal canker, caused by the fungus *Biscogniauxia mediterranea*.

This disease is characterized by necrotic cankers on host stems and branches (Henriques *et al.*, 2016). When plants are under stress, *B. mediterranea* can rapidly colonize xylem and bark tissues, resulting in the production of large cankers. These accelerate decline and eventually cause tree death. Ascospores (the main *B. mediterranea* inoculum) are widely disseminated via airborne dispersal and insect transmission (Henriques *et al.*, 2014).

Genetic diversity within *B. mediterranea* populations from single stromata, and from different hosts and geographic locations, has been related to high rates of sexual reproduction and the heterothallic mating system of *B. mediterranea* (Vannini *et al.*, 1999; Henriques *et al.*, 2014). This pathogen has been reported in other woody hosts, including *Erica multiflora* (Yangui *et al.*, 2019), *Olea europaea* (Gharbi *et al.*, 2020) in Tunisia, and *Amygdalus scoparia* in Iran (Rostamian *et al.*, 2016). However, *B. mediterranea* has not been previously recorded in *Prunus dulcis*.

The genetic heterogeneity and biology of *B. mediterranea,* and current climatic change scenarios, are factors that could favour pathogen occurrence and spread to new hosts. The main goal of the present research was to confirm almond (*Prunus dulcis*) as a new host of *B. mediterranea*, and to morphologically and molecularly characterize this pathogen.

MATERIALS AND METHODS

During a survey conducted in March 2022, several symptomatic almond trees with severe orange/black exudates on trunks were observed in an almond orchard in Beja, Portugal (Figure 1, A and B). Samples were collected from branches and trunks of three symptomatic trees each of Vairo and Soleta cultivars. These samples were fractionated into small fragments, and were then

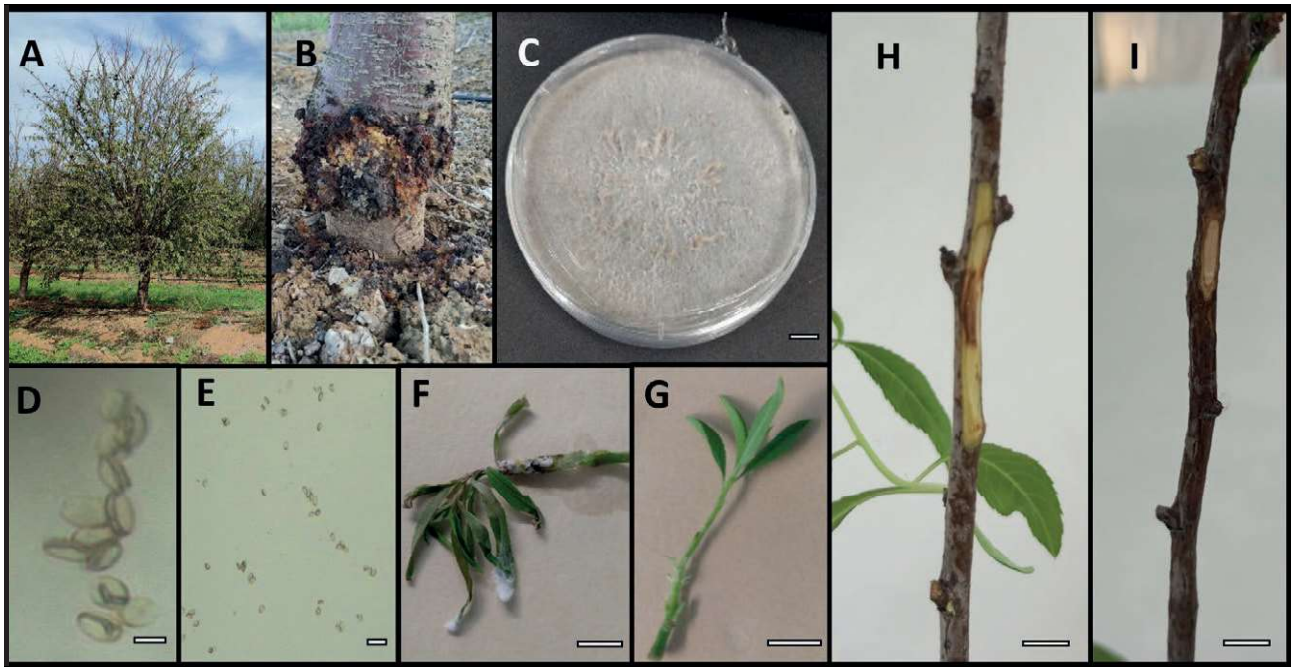

Figure 1. Aspects of a diseased almond tree, *Biscogniauxia mediterranea* morphology, and pathogenicity testing. (**A**) Symptomatic almond tree (*Prunus dulcis*). (**B**) Detail of an almond tree with severe orange and black exudate on the trunk. (**C**) Colony of *B. mediterranea* isolated from the almond tree, and growing in Petri dish containing PDA. (**D** and **E**) Microscope images of conidia. (**F**) Image of an *in vitro*-grown almond shoot, 1 week after inoculation with a *B. mediterranea* isolate. (**G**) Control *in vitro*-grown almond shoot 1 week after wounding and inoculation with a sterile PDA plug. (**H**) Almond tree stem with a necrotic lesion, 1 month after inoculation with a *B. mediterranea* isolate. (I) Control almond tree stem 1 month after wounding and inoculation with a sterile PDA plug. (Scale bars: 1 cm in C, F, G, H, and I; 40 μm in E; 100 μm in D).

surface disinfected to suppress epiphytic microorganisms (Varanda *et al.*, 2016). Surface disinfection included a series of 3 min immersions in 96% ethanol, 3% sodium hypochlorite solution, 70% ethanol, and then ultra-pure water, under a sterile laminar airflow environment. The samples were then incubated on Potato Dextrose Agar (PDA; VWR Portugal), in Petri dishes, at 25ºC for 5 d. Resulting colonies were sub-cultured to fresh PDA, and were then incubated (as above) for 10 d. Microscope examinations were carried out using a Leica DM750 light microscope, and relevant images were captured using a Leica ICC50W digital camera and Leica Application Suite LAZ EZ v. 3.4.0 software.

To obtain genomic DNA, fungal mycelium was suspended in 1× TE (100× Stock solution) (Sigma), then frozen at -20ºC for 3 min., thawed at 75ºC, and the vortexed for 2 min. These steps were repeated three times. The final thawing was extended to 15 min, followed by a 5 min centrifuge spin at 15700 g (Paiva, 2011).

For PCR amplification, the internal transcribed spacer (ITS) region was targeted with primer pair ITS-1/ITS-4 (White *et al.*, 1990), and the amplified fragments were sequenced by Sanger (STABVIDA, Portugal). The sequences obtained were analyzed using the BioEdit Sequence Alignment Editor v.7.2.3 (Hall, 1999), and were compared with homologous sequences deposited in the National Center for Biotechnology Information (NCBI) platform.

Biscogniauxia mediterranea isolates P.V.Fs.R3.3 (GenBank ID OR908437), P.S.Hs.R1.1 (GenBank ID PQ728784), P.V.Hs.R2.4 (GenBank ID PQ728785), P.V.Hs.R3.1 (GenBank ID PQ728786), and P.S.Hs.R2.4 (GenBank ID PQ728787) from the present study were selected for molecular characterization using a second primer pair targeting the translation elongation factor, EF1-728F/EF1-986R, as described by Carbone and Kohn (1999). Phylogenetic analyses were carried out using sequences of *Biscogniauxia* spp. deposited in GenBank (NCBI). The DNA sequences were aligned using the BioEdit software, and the alignments were concatenated in NEXUS format with partitions for each gene using the SequenceMatrix program v. 1.7.8 (Vaidya *et al.*, 2011). The phylogenetic tree was constructed using the maximum likelihood method in IQ-TREE (Nguyen *et al.*, 2015), with automatic selection of evolutionary models and support calculated by 1000 bootstrap replications. The tree was visualized and annotated in the iTOL v7 (Letunic and Bork, 2024).

Isolate P.V.Fs.R3.3 of *B. mediterranea* was used for pathogenicity tests. A preliminary assay was carried out by inoculating ten shoots of an *in vitro*-growing bitter almond cultivar each with a mycelium plug harvested from actively-growing colonies on PDA. The inoculum was applied to stems that were previously wounded with scalpel-made cuts. Assessments were made 1 week after *in vitro* inoculations. A second experiment was carried out by inoculating ten *ex-vitro* almond trees (commercially purchased 1-year-old potted plants) with isolate P.V.Fs.R3.3. All inoculated material was collected from potted plants after 1 month, and the lengths of wood discolouration from the inoculation points were measured. In these experiments, the inoculation controls were ten shoots of *in vitro*-grown plants, or ten potted plants that were similarly wounded, but were inoculated with sterile PDA plugs. To determine fulfillment of Koch's postulates, fungal re-isolations were made from the edges of necrotic lesions (as described above), and fungal identities were confirmed using colony morphology and ITS region sequencing. Statistical significance of variations in mean lesion lengths from the second pathogenicity assay was assessed using Statistica 7's one-way analysis of variance (ANOVA) (StatSoft, Inc., 2004).

RESULTS AND DISCUSSION

Analyses of results obtained for both cultivars (three trees of each cultivar) demonstrated that *B. mediterranea* was the main identified fungus (32%) amongst 87 isolates obtained, followed by *Alternaria* spp. (21%), *Fusarium* spp. (18%), and *Trichoderma* spp. (13%).

The BLASTn analysis of the sequence obtained from isolate P.V.Fs.R3.3 showed 99.82% nucleotide for ITS region similarity with the *B. mediterranea* reference strain GenBank ID JQ781799 from Portugal, and 99.68% TEF-1 similarity with GenBank ID MZ221965. Morphology of colonies on PDA and conidia of the isolates identified as *B. mediterranea* was assessed (Figure 1, C, D, and E). As described by Henriques *et al.* (2014), the colour of the aerial mycelium of colonies on PDA varied from white to grayish, to smoke grey. Some isolates had brownish colouration, eventually producing dark exudates. Isolate P.V.Fs.R3.3 (GenBank ID OR908437) produced white colonies on PDA (Figure 1 C).

The phylogenetic analyses demonstrated the similarity of the sequences with other reported sequences of *B. mediterranea* (Figure 2). All the isolates used in this study are in the same cluster as the other *B. mediterranea* isolates and are separated from the other *Biscogniauxia* spp., except for *B. rosacearum*, which is in the same cluster as *B. mediterranea*. This can be explained by the proximity of *B. rosaceaum* to *B. mediterranea* already described in previous studies considering morphological, cultural, and molecular data (Raimondo

Table 1. Isolates (species and codes), hosts, origins, and GenBank accession numbers of the *Biscogniauxia* species and strains used in phylogenetic analyses.

Species	Isolate ID	Host	Origin	GenBank accession number ITS	GenBank accession number TEF-1
Biscogniauxia mediterranea	Bm04.001	*Quercus suber*	Portugal	KM216752	KM216788
	Bm06.003	*Quercus suber*	Morocco	KM216753	KM216789
	Bm07.003	*Quercus suber*	Portugal	KM216754	KM216790
	Bm09.001	*Quercus suber*	Tunisia	KM216755	KM216791
	Bm10.006	*Quercus suber*	Portugal	KM216757	KM216793
	Bm10.012	*Quercus rotundifolia*	Portugal	KM216758	KM216794
	Bm10.016	*Quercus suber*	Italy	KM216759	KM216795
	Bm12.005	*Quercus suber*	Portugal	KM216766	KM216802
	Bm12.015	*Quercus suber*	Tunisia	KM216769	KM216805
	Bm12.022	*Quercus suber*	Portugal	KM216771	KM216807
	Bm12.023	*Eucalyptus globulus*	Portugal	KM216772	KM216808
	Bm12.024	*Quercus suber*	Portugal	KM216774	KM216810
	Bm12.039	*Quercus suber*	France	KM216780	KM216816
	Bm13.004	*Quercus suber*	France	KM216781	KM216817
	Bm13.007	*Quercus suber*	Algeria	KM216782	KM216818
	P.S.Hs.R1.1	*Prunus dulcis*	Portugal	PQ728784	PV963833
	P.V.Hs.R2.4	*Prunus dulcis*	Portugal	PQ728785	PV963834
	P.V.Hs.R3.1	*Prunus dulcis*	Portugal	PQ728786	PV963835
	P.S.Hs.R2.4	*Prunus dulcis*	Portugal	PQ728787	PV963836
	P.V.Fs.**R3.3**	***Prunus dulcis***	**Portugal**	**OR908437**	**PV963837**
Biscogniauxia formosana	*B. formosana*	Bark of *Quercus* sp.	Taiwan	JX507802	-
Biscogniauxia atropunctata	*B. atropunctata*	Wood	USA	JX507799	-
Biscogniauxia maritima	*B. maritima*	Plant leaf	South Korea	MT269517	-
Biscogniauxia arima	*B. arima* EF026150	Wood	Mexico	EF026150	.
Biscogniauxia rosacearum	*B. rosacearum*	*Prunus dulcis*	Iran	MZ190891	-
Biscogniauxia mummularia	*B. mummularia*	*Fagus sylvaticus*	Italy	AJ246230	-
Biscogniauxia anceps	*B. anceps*	Wood	Spain	OQ990096	-
Outgroups					
Xylaria hypoxylon	*Xylaria hypoxylon*	*Fagus sylvatica*	Germany	AM993138	-

et al., 2016; Sohrabi *et al.*, 2022). A similar result was observed in our phylogenetic analysis, where *B. rosacearum* clustered together with all *B. mediterranea* isolates and with high genetic similarity to our isolate P.S.Hs. R1.1. Between our isolates obtained from almond trees, slight differences in genetic sequences were observed, although the isolates P.V.Hs.R2.4, P.V.Fs.R3.3, and P.S.Hs.R2.4 were genetically identical.

In the preliminary assay, the ten *in vitro* plants inoculated with *B. mediterranea* developed superficial and internal brown-black discolourations (Figure 1 F), with white mycelium growing from the points of inoculation to the stems and leaves 1 week after inoculations. *Biscogniauxia mediterranea* was then re-isolated from the lesions from all the inoculated plants, and grew on PDA with a 100% re-isolation rate, confirming Koch's postulates and demonstrating pathogenicity of *B. mediterranea* to almond trees (*Prunus dulcis*). The *in vitro* control plants presented healed wounds without any disease symptoms (Figure 1 G). In the second pathogenicity assay, using potted almond trees inoculated with *B. mediterranea*, internal wood discolourations were observed in nine of the ten inoculated almond trees at 1 month after inoculation. These lesions varied in length from 0.3 cm to 8.8 cm (mean = 2.18 cm.), upward and downward from the points of inoculation (Figure 1 H). The control almond trees presented healed wounds without any wood discolouration (Figure 1 I). Statistically significant differences ($P \leq 0.013$) were detected between plants inoculated with *B. mediterranea* and the non-inoculated controls, where no pathogen re-isolations were detected. For re-isolation from material inoculated with *B. mediterranea*,

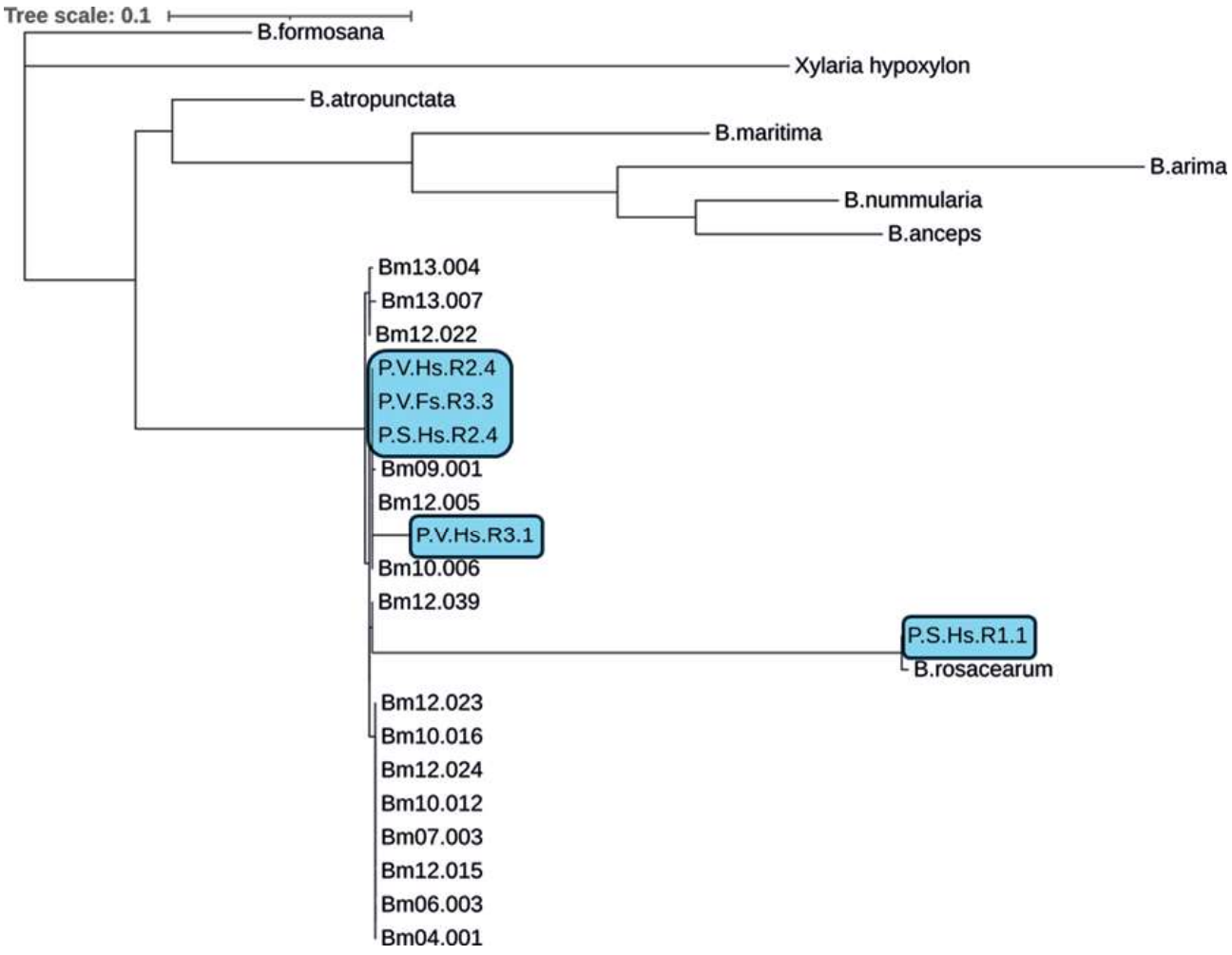

Figure 2. Phylogenetic tree based on Bayesian Inference analyses of combined ITS and TEF-1 sequence data. Almond tree isolates obtained in the present study are shown in blue boxes. The scale bar shows expected changes per site.

the fungi *Boeremia exigua* (5%), *Diaporthe* sp. (5%), *Didymella pomorum* (20%), *Epicoccum nigrum* (5%), *Wilsonomyces carpophilus* (5%), *Alternaria alternata* (10%), *Neofusicoccum parvum* (5%), and *Botryosphaeria dothidea* (5%) were isolated. Inoculation controls yielded similar re-isolation proportions of these fungi, including *Boeremia exigua* (8%), *Diaporthe* sp. (4%), *Didymella pomorum* (20%), *Alternaria alternata* (14%), *Neofusicoccum parvum* (8%), *Botryosphaeria dothidea* (8%), and other species. Despite the necrotic lesions observed on woody stems, we speculate that the phytosanitary status of the almond trees may have influenced the rates of re-isolation of *B. mediterranea* in the second pathogenicity assay.

In conclusion, identification of isolate P.V.Fs.R3.3, based on morphological and molecular data (obtained by sequencing the ITS and TEF-1 genes), has demonstrated that almond trees are a new host for *B. mediterranea* in Portugal, and probably elsewhere. Furthermore, the pathogenicity tests showed that *B. mediterranea* can cause interior wood discolouration lesions in almond plants. The high genetic variability in this fungus is likely to allow it to adapt to new hosts and environmental conditions, demonstrating the importance of edaphoclimatic conditions to the phytosanitary status of potential host plants. Further research is required to determine the impacts of *B. mediterranea* on the health, productivity, and longevity of almond orchards.

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