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Diversity of *Botryosphaeriaceae* species associated with canker and dieback of avocado (*Persea americana*) in Italy

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Summary. Increased branch canker and dieback were observed in commercial avocado (*Persea americana*) orchards in Sicily, Italy. Surveys were conducted in 2021 and 2022 on 11 orchards to investigate etiology of the disease. Seventy-five plants from four orchards, showing branch canker and dieback, were sampled. Isolations from woody diseased tissues revealed the presence of fungi (*Botryosphaeriaceae*). Identification of the isolates was achieved by morphological and multi-loci phylogenetic analyses (Maximum Parsimony and Maximum Likelihood) of the ITS, *tef1*-α, and *tub2* loci. *Botryosphaeria dothidea, Lasiodiplodia citricola*, *Macrophomina phaseolina, Neofusicoccum cryptoaustrale*, and *Neofusicoccum luteum* were identified. Representative isolates collected from the orchards, characterized based on the *tub2* locus and identified as *N. parvum,* were excluded from this study, since this species has already been reported in our territory. Pathogenicity tests were conducted on potted, asymptomatic, 2-year-old avocado trees using mycelial plugs. These tests showed that all the *Botryosphaeriaceae* species characterized in this study were pathogenic to avocado. This is the first report of *L. citricola*, *M. phaseolina* and *N. cryptoaustrale* causing canker and dieback on avocado trees, and is the first record of these fungi causing branch disease on avocado in Italy.

Keywords. Fungal diseases, *Botryosphaeria*, *Lasiodiplodia*, *Macrophomina*, *Neofusicoccum*, phylogeny.

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INTRODUCTION

Avocado (*Persea americana* L.) is a tree native to Mexico and has spread to many tropical and subtropical regions (Bost *et al*., 2013). Consumption of avocado fruit and new plantings of avocados has considerably increased (Bost *et al*., 2013). The greatest production is in Mexico, followed by Colombia and the Dominican Republic (FAOSTAT, 2022). In Europe, Spain was the first country to develop commercial production of avocados (Pérez-Jiménez, 2008). In Italy, avocado production is spread in the Southern regions, mainly in Sicily, where the cultivated area has increased in the last 10 years (Migliore

et al., 2017). In Sicily, avocado provides good agricultural diversification as an alternative crop to citrus (Guarnaccia *et al*., 2016).

Several diseases can affect avocados, and several fungi taxa have been associated with different symptoms. Traditionally, root diseases have been considered the most important limiting factors for avocado production. Among these, those caused by *Phytophthora cinnamomi* and *Rosellinia necatrix* are considered the most important and widespread diseases of avocado, leading to serious losses, especially in the Mediterranean regions where avocado production is well established (Zentmyer, 1980; López-Herrera and Melero-Vara, 1992; Fiorenza *et al*., 2021). In recent years, species of *Nectriaceae* have also been shown to be important, especially in Australia where different taxa have been associated with crown root rot disease (Parkinson *et al*., 2017). In Italy, recent studies have shown the presence of *Nectriaceae* spp. causing a complex of root symptoms (Vitale *et al*., 2012; Aiello *et al*., 2020b).

In recent decades, increased research has been carried out on canker diseases of fruit and nut crops (Moral *et al*., 2019; Guarnaccia *et al*., 2022a). These diseases have been re-discovered as important and limiting for perennial crops, especially because they cause polyetic epidemics, a complex of pathogen taxa are involved, and most of the causal agents are polyphagous and live as latent pathogens. Among the taxa associated and responsible for shoot, branch and trunk cankers and dieback, *Botryosphaeriaceae* is a widely investigated group of fungi (Batista *et al.,* 2021). *Botryosphaeriaceae* includes fungi that can be pathogens, saprobes and endophytes (Slippers and Wingfield, 2007; Phillips *et al*., 2013), and can be severe threats to fruit, nut, ornamental and forest trees (Slippers and Wingfield, 2007; Moral *et al*., 2019). DNA-based tools, especially multi-locus phylogeny, have shown that many genera and species within the *Botryosphaeriales* have been described, synonymized, and re-accommodated (Zhang *et al*., 2021).

On avocado, despite sporadic reports of *Diaporthe* species associated with cankered tissues (Guarnaccia *et al*., 2016; Torres *et al*., 2016; Mathioudakis *et al*., 2020), different *Botryosphaeriaceae* have been extensively reported worldwide causing canker and dieback on woody tissues and fruit rots, including: *Botryosphaeria dothidea*, *Diplodia aromatica, D. dominicana, D. mutila*, *D. pseudoseriata*, *D. seriata*, *Dothiorella iberica, Lasiodiplodia laeliocattleyae*, *L. pseudotheobromae, L. theobromae*, *Neofusicoccum australe*, *N. luteum, N. mangiferae, N. mediterraneum, N. nonquaesitum*, *N. parvum*, and *N. stellenboschiana* (Peterson, 1978; Hartill, 1991; Hartill and Everett, 2002; Zea-Bonilla *et al*., 2007; McDonald *et al*., 2009; Ni *et al*., 2009; McDonald and Eskalen, 2011; Ni *et al*., 2011; Dann *et al*., 2013; Auger *et al*., 2013; Twizeyimana *et al*., 2013; Carrillo *et al*., 2016; Valencia *et al*., 2019; Arjona-Girona *et al*., 2019; Tapia *et al*., 2020; Guarnaccia *et al*., 2020; Wanjiku *et al*., 2020; Qiu *et al*., 2020; Rodríguez-Gálvez *et al*., 2021; Avenot *et al*., 2022). On avocado, canker and dieback symptoms can appear on shoots, branches, and trunks. Usually, reddish sap that became white/beige with the age has been associated with cankers. The tree bark can be friable or sunken and necrotic, showing cracking, with external dark discolouration. Internally, infected wood becomes brown with characteristic wedge-shaped discolourations affecting the xylem. Under high disease pressure, severe xylem colonization may be observed, with associated wilting of shoots and leaves, that remain attached.

In Italy, the first investigations of avocado branch and trunk canker were reported in 2016, showing the presence of *Botryosphaeriaceae* (*N. parvum*), *Diaporthaceae* (*D. foeniculacea* and *D. sterilis*) and *Glomerellaceae* (*Colletotrichum gloeosporioides* and *C. fructicola*) (Guarnaccia *et al*., 2016). Studies on avocado canker diseases in Italy have continued, and in 2018, a new species *Neocosmospora persea* was described, causing branch and trunk canker, which was also later reported in Greece (Guarnaccia *et al*., 2018, 2022b). More recently, the new species *Neopestalotiopsis siciliana* and *Ne. rosae* were reported as causing stem lesions and dieback on avocado (Fiorenza *et al*., 2021).

An increased incidence of shoot and branch canker has been observed in Sicilian avocado orchards since 2016. The present study has investigated the diversity of *Botryosphaeriaceae* associated with symptomatic trees. The aims of the study were: (i) to characterize the *Botryosphaeriaceae* recovered from symptomatic avocado samples, and (ii) to test their pathogenicity to this host.

MATERIALS AND METHODS

Field surveys and fungal isolation

Surveys were conducted in Sicily (Italy) during 2020 and 2021 in the main avocado production areas (Catania, Messina, and Siracusa provinces). Eleven orchards were investigated and selected for sampling. Samples (three to ten plants from each site) of symptomatic branches, trunks and shoots were collected, and brought to the Plant Pathology laboratory, Dipartimento di Agricoltura, Alimentazione e Ambiente, Sezione di Patologia Vegetale, University of Catania. Small sections (0.5 cm^2) of symptomatic tissues were surface disinfected for 1 min in 1.5% sodium hypochlorite solution (NaOCl),

rinsed in sterile distilled water, dried on sterile absorbent paper and placed on potato dextrose agar (PDA; Lickson) amended with 100 mg L^{-1} of streptomycin sulphate (Sigma-Aldrich) to prevent bacterial growth, and incubated at 25±1°C for 7 d. Isolation frequency of *Botryosphaeriaceae* was calculated using the formula: F = $(N_{Bot}/N_{Tot}) \times 100$, where F is the frequency of *Botryosphaeriaceae*; N_{Bot} is the number of woody fragments from which *Botryosphaeriaceae* were isolated; and N_{Tot} is the total number of woody fragments from which fungi were isolated. Single hyphal tip cultures on PDA were obtained. Thes isolates are maintained in the Plant Pathology collection of the University of Catania.

Morphological and culture characters of isolates

Representative isolates of each morphologically different group of isolates were transferred onto Technical Agar (AT, 1.2% Agar Technical, Biolife) supplemented with autoclaved pine needles (Smith *et al*., 1996), and were incubated at room temperature under UV light. The size, colour, and shape of conidia produced by the isolates were examined. After 14 d, pycnidia were observed with a stereoscope, and were mounted in 100% lactic acid. Fifty conidia from each representative isolate were measured (length and width), using an Olympus-BX61 fluorescence microscope coupled to an Olympus DP70 digital camera. Measurements were captured using the software analySIS 3.2 (Soft Imaging System GmbH). Dimensions are reported here as averages.

DNA extraction and PCR analyses

The representative isolates were cultivated on PDA for 7 d, and genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation) following the manufacture's protocol. The quality of the DNA was determined using a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific), and was diluted to 5 ng μL^{-1} with nuclease-free water. The internal transcriber spacer region (ITS) of the nuclear ribosomal RNA operon was amplified with primers ITS5 and ITS4 (White *et al*, 1990), and the primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used to amplify part of the translation elongation factor 1 alpha locus (*tef1*-α), and primer sets Bt2a and Bt2b (Glass and Donaldson, 1995) were used for the partial beta tubulin locus (*tub2*). The amplifications were each carried out in a final volume of 25 µL using One Taq® 2× Master Mix with Standard Buffer (BioLabs), according to the manufacturer's instructions, on an Eppendorf Mastercycler (AG 22331). The PCR consisted of initial 30 s at 94°C, followed by 35 cycles at 94°C for 30 s, 50–52°C (ITS), 57–59°C (*tef1-α*), or 52°C (*tub2*) for 1 min, followed by 68°C for 1 min, and 5 min at 68°C. All PCR products were visualized on 1% agarose gels (90 V for 40 min), stained with GelRed®, purified, and sequenced by Macrogen Inc. Forward and reverse DNA sequences were assembled and edited using AliView software (Larsson, 2014), and were submitted to GenBank. Sixty-two isolates were sequenced (amplifying the *tub2* locus only), and based on these preliminary results only 23 representative isolates were considered for further locus sequencing and phylogenetic analyses.

Phylogeny

Sequences were read, assembled, and edited using MEGAX: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar *et al*., 2018). The ITS, *tub2* and *tef1*-α DNA sequence datasets were aligned using MEGAX. For comparison, 57 additional sequences were selected according to the most recent taxonomic classification of *Botryosphaeriaceae* genera and species involved in this study (Table 1). Two analyses were performed. Maximum parsimony analysis (MP) was performed in PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0a (Swofford, 2002). The analysis of the combined dataset (ITS + *tub2* + *tef1*-α) was obtained with the heuristic search function and tree bisection and reconstruction (TBR) as branch swapping algorithms with the branch swapping option set on 'best trees' only. Gaps were treated as 'missing', the characters were unordered and of equal weight, and Maxtrees were limited to 100. Tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated. A total of 1000 bootstrap replicates were performed to test the robustness of the tree topologies. The best-fit model of nucleotide evolution for each locus, according to the Akaike information criterion (AIC), was evaluated using MrModeltest v. 2.4 (Nylander, 2004). The Maximum Likelihood analysis (ML) of the combined loci was performed in GARLI v.0.951 (Zwickl, 2006), and clade support was assessed by 1000 bootstrap replicates. *Phyllosticta ampelicida* (CBS 111645) and *Phyllosticta citricarpa* (CBS 102374) served as the outgroup taxa in both analyses.

Pathogenicity test

A pathogenicity test was conducted in a greenhouse, February to April 2022. Five 2-year-old asymptomatic avocado plants 'Hass' grafted on 'Zutano' rootstocks

Table 1. Information of fungal isolates used in the phylogenetic analysis and their corresponding GenBank accession numbers. Isolates in bold font are from this study. The "T" superfix identifies type material.

Species	Isolate ID	Host	Country	GenBank accession No.		
				ITS	*tef1-α*	*tub2*
Botryosphaeria agaves	CBS 133992 = MFLUCC 11-0125^{T}	*Agave* sp.	Thailand	JX646791	JX646856	JX646841
Botryosphaeria agaves	CBS 141505 = CPC 26299	*Agave* sp.	France	KX306750	MT592030	MT592463
Botryosphaeria corticis	CBS 119047^{T} CAP 197	*Vaccinium corymbosum*	New Jersey, USA	DQ299245	EU017539	EU673107
Botryosphaeria corticis	CBS 119048 = CAP 198	*Vaccinium corymbosum*	New Jersey, USA	DQ299246	EU017540	MT592464
Botryosphaeria dothidea	CBS 115476 = CMW 8000^{T}	*Prunus* sp.	Switzerland	AY236949	AY236898	AY236927
Botryosphaeria dothidea	CBS 110302 = CAP 007	*Vitis vinifera*	Portugal	AY259092	AY573218	EU673106
Botryosphaeria dothidea	**AB2**	***Persea americana***	**Catania, Sicily, Italy**	**OP654490**	**OP764459**	**OP764436**
Botryosphaeria dothidea	**AB4**	***Persea americana***	**Catania, Sicily, Italy**	**OP654491**	**OP764460**	**OP764437**
Botryosphaeria dothidea	**AB5**	***Persea americana***	**Catania, Sicily, Italy**	**OP654492**	**OP764461**	**OP764438**
Botryosphaeria dothidea	**AC5**	***Persea americana***	**Catania, Sicily, Italy**	**OP654493**	**OP764462**	**OP764439**
Botryosphaeria dothidea	**AC7**	***Persea americana***	**Catania, Sicily, Italy**	**OP654494**	**OP764463**	**OP764440**
Botryosphaeria dothidea	**AC9**	***Persea americana***	**Catania, Sicily, Italy**	**OP654495**	**OP764464**	**OP764441**
Botryosphaeria dothidea	**AC10**	***Persea americana***	**Catania, Sicily, Italy**	**OP654496**	**OP764465**	**OP764442**
Botryosphaeria dothidea	**AC11**	***Persea americana***	**Catania, Sicily, Italy**	**OP654497**	**OP764466**	**OP764443**
Botryosphaeria fabicerciana	CBS 118831 = CMW 14009	*Syzygium cordatum*	South Africa	DQ316084	MT592032	MT592468
Botryosphaeria fabicerciana	CBS 127193 = CMW 27094^{T}	*Eucalyptus* sp.	China	HQ332197	HQ332213	KF779068
Botryosphaeria kuwatsukai	CGMCC 3.18007^{T}	*Malus* sp.	China	KX197074	KX197094	KX197101
Botryosphaeria kuwatsukai	CGMCC 3.18008	*Amygdalus* sp.	China	KX197075	KX197095	KX197102
Botryosphaeria qingyuanensis	CERC 2946 = CGMCC 3.18742^{T}	*Eucalyptus hybrid*	China	KX278000	KX278105	KX278209
Botryosphaeria qingyuanensis	CERC 2947 = CGMCC 3.18743	*Eucalyptus hybrid*	China	KX278001	KX278106	KX278210
Botryosphaeria ramosa	CERC 2001 = CGMCC 3.187396	*Eucalyptus hybrid*	China	KX277989	KX278094	KX278198
Botryosphaeria ramosa	CBS 122069 = CMW 26167^{T}	*Eucalyptus camaldulensis*	Australia	EU144055	EU144070	KF766132
Lasiodiplodia citricola	CBS 124706 = IRAN 1521C	*Citrus* sp.	Iran	GU945353	GU945339	KU887504
Lasiodiplodia citricola	CBS 124707 = IRAN 1522C^{T}	*Citrus* sp.	Iran	GU945354	GU945340	KU887505
Lasiodiplodia citricola	CGMCC 3.19022	*Vaccinium corymbosum*	China	MH330318	MH330327	MH330324
Lasiodiplodia citricola	**AC20**	***Persea americana***	**Catania, Sicily, Italy**	**OP654498**	**OP764481**	**OP764444**
Lasiodiplodia euphorbiaceicola	CMM 3609^{T}	*Jatropha curcas*	Brazil	KF234543	KF226689	KF254926
Lasiodiplodia euphorbiaceicola	CMW 33268	*Adansonia* sp.	Senegal	KU887131	KU887008	KU887430
Lasiodiplodia mahajangana	CBS 124925 = CMW 27801^{T}	*Terminalia catappa*	Madagascar	FJ900595	FJ900641	FJ900630
Lasiodiplodia mahajangana	CBS 124926 = CMW 27818	*Terminalia catappa*	Madagascar	FJ900596	FJ900642	FJ900631
Lasiodiplodia mediterranea	CBS 137783 = BL 1^{T}	*Quercus ilex*	Italy	KJ638312	KJ638331	KU887521
Lasiodiplodia mediterranea	CBS 137784 = BL 101	*Vitis vinifera*	Italy	KJ638311	KJ638330	KU887522
Lasiodiplodia parva	CBS 456.78^{T}	*Cassava*	Colombia	EF622083	EF622063	KU887523
Lasiodiplodia parva	CBS 494.78	*Cassava*	Colombia	EF622084	EF622064	EU673114

(Continued)

Table 1. (Continued). Isolates in bold font are from this study. The "T" superfix identifies type material.

Species	Isolate ID	Host	Country	GenBank accession No.		
				ITS	*tef1-α*	*tub2*
Lasiodiplodia viticola	CBS 128313 = UCD 2553AR^T	*Vitis vinifera*	USA	HQ288227	HQ288269	HQ288306
Lasiodiplodia viticola	CBS 128314 = UCD 2604MO	*Vitis vinifera*	USA	HQ288228	HQ288270	HQ288307
Macrophomina euphorbiicola	CMM4134 / CCMF-CNPA 288^T	*Ricinus communis*	Brazil	KU058936	KU058906	MF457658
Macrophomina euphorbiicola	CMM4145 / CCMF-CNPA 289	*Ricinus communis*	Brazil	KU058937	KU058907	MF457659
Macrophomina phaseolina	CBS 227.33^T	*Zea mays*		KF531825	KF531804	KF531806
Macrophomina phaseolina	KARE1339	*Pistacia vera*	USA	MN097202	MN106057	MN106087
Macrophomina phaseolina	**AC28**	***Persea americana***	**Catania, Sicily, Italy**	**OP654499**	**OP764467**	**OP764445**
Macrophomina phaseolina	**AC29**	***Persea americana***	**Catania, Sicily, Italy**	**OP654500**	**OP764468**	**OP764446**
Macrophomina phaseolina	**AC51**	***Persea americana***	**Catania, Sicily, Italy**	**OP654501**	**OP764469**	**OP764447**
Macrophomina phaseolina	**AC52**	***Persea americana***	**Catania, Sicily, Italy**	**OP654502**	**OP764470**	**OP764448**
Macrophomina pseudophaseolina	CPC 21417	*Arachis hypogaea*	Senegal	KF951791	KF952153	KF952233
Macrophomina pseudophaseolina	CPC 21524	*Hibiscus sabdarifa*	Senegal	KF951799	KF952161	KF952240
Macrophomina tecta	BRIP 70781^T	*S. bicolor*	Chinchilla, Qld	MW591684	MW592271	MW592300
Macrophomina tecta	BRIP 71603	*S. bicolor*	Chinchilla, Qld	MW591631	MW592218	MW592301
Macrophomina vaccinii	CGMCC 3.19503^T	*V. corymbosum* × *V. darrowii*	China	MK687450	MK687426	MK687434
Macrophomina vaccinii	CGMCC 3.19504	*V. corymbosum* × *V. darrowii*	China	MK687451	MK687427	MK687435
Neofusicoccum australe	CBS 139662 = CMW 6837^T	*Acacia* sp.	Victoria, Australia	AY339262	AY339270	AY339254
Neofusicoccum australe	CBS 113220 = CMW 6853	*Sequoiadendron*	Australia	AY339263	AY339271	AY339255
Neofusicoccum cryptoaustrale	CBS 122813 = CMW 23785^T	*Eucalyptus* sp.	South Africa	FJ752742	FJ752713	FJ752756
Neofusicoccum cryptoaustrale	**AVORAM1**	***Persea americana***	**Catania, Sicily, Italy**	**OP654508**	**OP764476**	**OP764454**
Neofusicoccum cryptoaustrale	**AVORAM2**	***Persea americana***	**Catania, Sicily, Italy**	**OP654509**	**OP764477**	**OP764455**
Neofusicoccum cryptoaustrale	**AVORAM3**	***Persea americana***	**Catania, Sicily, Italy**	**OP654510**	**OP764478**	**OP764456**
Neofusicoccum cryptoaustrale	**AVORAM4**	***Persea americana***	**Catania, Sicily, Italy**	**OP654511**	**OP764479**	**OP764457**
Neofusicoccum cryptoaustrale	**AVORAM5**	***Persea americana***	**Catania, Sicily, Italy**	**OP654512**	**OP764480**	**OP764458**
Neofusicoccum lumnitzerae	CBS 139674 = CMW 41469^T	*Lumnitzera racemosa*	South Africa	KP860881	KP860724	KP860801
Neofusicoccum lumnitzerae	CBS 139675 = CMW 41228	*Lumnitzera racemosa*	South Africa	MT587480	MT592193	MT592685
Neofusicoccum luteum	CBS 110497 = CPC 4594 = CAP 037	*Vitis vinifera*	Portugal	EU673311	EU673277	EU673092
Neofusicoccum luteum	CBS 110299 = LM 926 = CAP 002^T	*Vitis vinifera*	Portugal	AY259091	KX464688	DQ458848
Neofusicoccum luteum	**AVF3**	***Persea americana***	**Catania, Sicily, Italy**	**OP654503**	**OP764471**	**OP764449**
Neofusicoccum luteum	**AVF5**	***Persea americana***	**Catania, Sicily, Italy**	**OP654504**	**OP764472**	**OP764450**
Neofusicoccum luteum	**AVF6**	***Persea americana***	**Catania, Sicily, Italy**	**OP654505**	**OP764473**	**OP764451**
Neofusicoccum luteum	**AVF7**	***Persea americana***	**Catania, Sicily, Italy**	**OP654506**	**OP764474**	**OP764452**
Neofusicoccum luteum	**AVF8**	***Persea americana***	**Catania, Sicily, Italy**	**OP654507**	**OP764475**	**OP764453**
Neofusicoccum mediterraneum	CBS 121558	*Olea europea*	Italy	GU799463	GU799462	GU799461
Neofusicoccum mediterraneum	CBS 121718 = CPC 13137^T	*Eucalyptus* sp.	Greece	GU251176	GU251308	GU251836

(Continued)

Table 1. (Continued). Isolates in bold font are from this study. The "T" superfix identifies type material.

Species	Isolate ID	Host	Country	GenBank accession No.		
				ITS	*tef1-α*	*tub2*
Neofusicoccum protearum	CBS 114176 = CPC 1775 = JT 189^{T}	*Leucadendron salignum* × *L. laureolum*	South Africa	AF452539	KX464720	KX465006
Neofusicoccum protearum	CBS 115177 = CPC 4357	*Protea magnifica*	South Africa	FJ150703	MT592239	MT592731
Neofusicoccum stellenboschiana	CBS 110864 = CPC 4598	*Vitis vinifera*	South Africa	AY343407	AY343348	KX465047
Neofusicoccum terminaliae	CBS 125263 = CMW 26679^{T}	*Terminalia sericea*	South Africa	GQ471802	GQ471780	KX465052
Neofusicoccum terminaliae	CBS 125264 = CMW 26683	*Terminalia sericea*	South Africa	GQ471804	GQ471782	KX465053
Neofusicoccum ursorum	CBS 122811 = CMW 24480^{T}	*Eucalyptus* sp.	South Africa	FJ752746	FJ752709	KX465056
Neofusicoccum ursorum	CBS 122812 = CMW 23790	*Eucalyptus* sp.	South Africa	FJ752745	FJ752708	KX465057
Neofusicoccum viticlavatum	CBS 112878 = CPC 5044 = JM 86^{T}	*Vitis vinifera*	South Africa	AY343381	AY343342	KX465058
Neofusicoccum viticlavatum	CBS 112977 = STE-U 5041	*Vitis vinifera*	South Africa	AY343380	AY343341	KX465059
Phyllosticta ampelicida	CBS 111645	*Taxus baccata*	Nethherlands	FJ824766	FJ824773	FJ824779
Phyllosticta citricarpa	CBS 102374	*Citrus aurantium*	Brazil	FJ824767	FJ538371	FJ824778

were selected for each tested fungal species. Inoculations were each carried out using a mycelium plug (0.5 cm^2) from a 10-d-old culture of each of *Botryosphaeria dothidea* (AC7), *Lasiodiplodia citricola* (AC20), *Macrophomina phaseolina* (AC29), *Neofusicoccum cryptoaustrale* (AVORAM4), and *Neofusicoccum lutem* (AVF5). Each inoculation site was first surface disinfected with a 70% ethanol solution. Two points of inoculation for each plant were made on the stem after removing a piece of bark with a sterile scalpel blade, placing the isolate mycelium plug onto the wound and covering it with Parafilm® (American National Can) to prevent desiccation. Three 2-year-old asymptomatic avocado plants were inoculated with sterile PDA plugs to serve as inoculation controls. The plants were moved to a greenhouse and regularly watered. Temperature in the greenhouse ranged from 18 to 27°C and humidity from 70 to 80%. The inoculated plants were monitored weekly for symptom development, and a final assessment was conducted 63 d after the inoculations. Lesion length measurements were recorded, and were statistically analyzed in Statistix 10 (Analytical Software, 2013) using analysis of variance (ANOVA). Mean differences were compared with the Fisher's protected least significant difference (LSD) test at $\alpha = 0.05$. To fulfill Koch's postulates, re-isolations were carried out following the procedure described above, and each re-isolated fungus was identified through observation of morphological characteristics.

RESULTS

Field surveys and fungal isolations

Disease was observed on 2 to 10-year-old avocado plants 'Hass', grafted on different rootstock cultivars ('Zutano', 'Duke 7', and 'Dusa') in Sicily (Italy). All the sampled plants showed symptoms of shoot and branch canker, and dieback emerging within the green canopies (Figure 1 A to D). Occasionally, a white powder was present on the surfaces of the lesions (Figure 1 E). It was also possible to observe the infections starting from pruning wounds (Figure 1 F and G). The bark of cankered shoots was cracked, darkly discoloured, and/or slightly sunken (Figure 1 H). Cankers were reddish-brown under the bark, and variable in shape. Necrotic lesions and internal discolouration were observed at the grafting points of young plants (Figure 1 I). Isolations frequently (41%) yielded Botryosphaeriaceae-like fungi, and *Botryosphaeriaceae* were detected in all the samples analyzed.

A total of 106 *Botryosphaeriaceae* isolates were collected and stored. Of these, 62 isolates (59%) were processed for DNA extraction, PCR, and sequencing. A

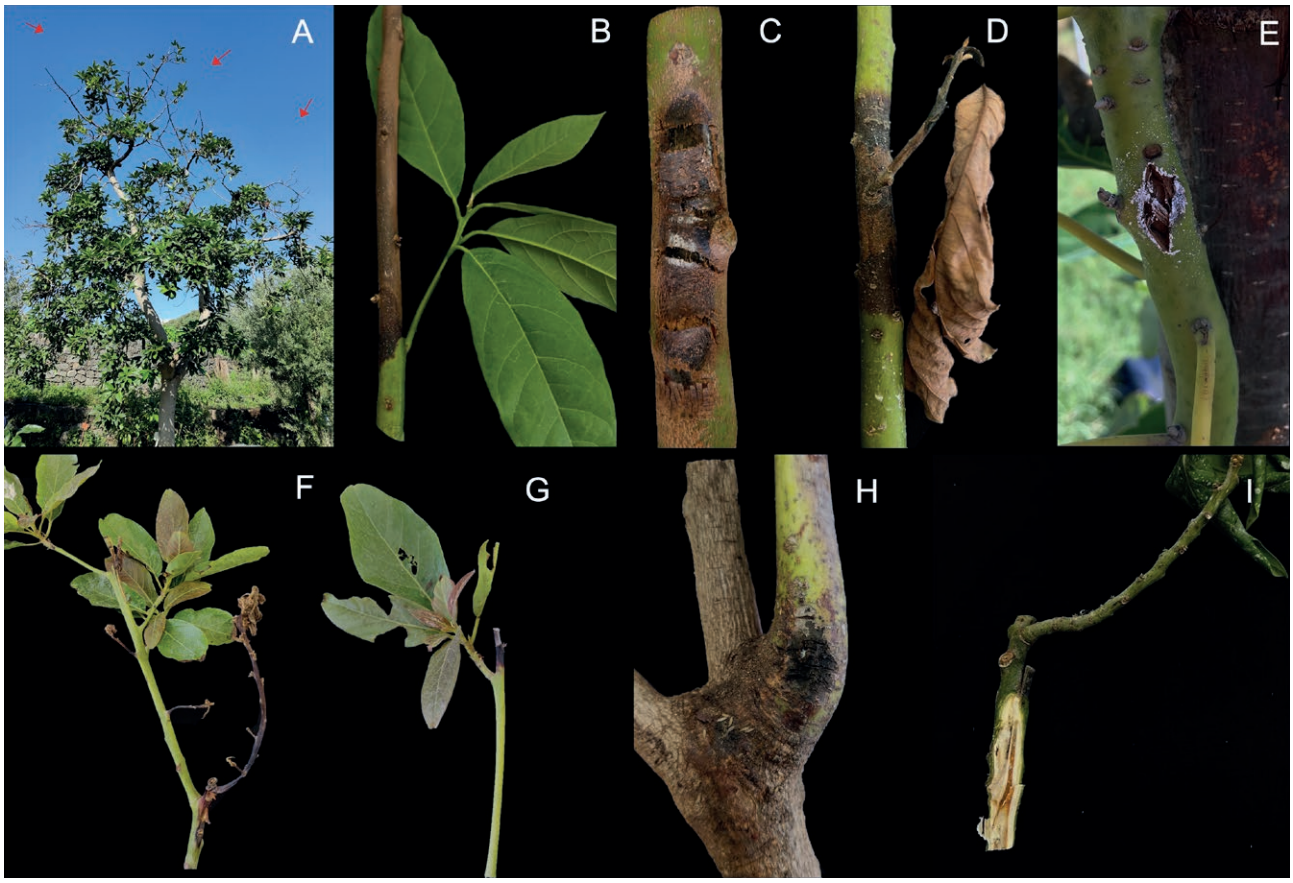

Figure 1. Symptoms of *Botryosphaeriaceae* on avocado trees observed in the field. **A**, Shoot dieback in the host canopy. **B**, Branch dieback. **C** and **D**, External canker (shoot canker). **E**, Canker with white powdery exudation. **F** and **G**, Infection originating from pruned wounds. **H**, bark cracking. **I**, infected grafting point.

preliminary screening based on the *tub2* locus was conducted on all 62 isolates, and this showed that representative isolates from seven orchards (orchard numbers 5 to 11, Table 2) were *N. parvum*. Since this fungal species was already characterized and reported in a preliminary study (Guarnaccia *et al*., 2016), these isolates were excluded from further locus sequencing and phylogenetic analyses, but the *N. parvum* isolates were collected and stored, since the present investigation showed that this fungus predominated in Sicilian avocado orchards. A total of 23 isolates derived from four orchards (orchards numbers 1 to 4) were fully characterized, as these isolates were previously unreported in Italy. These 23 isolates were from 75 young (2 to 4-year-old) plants showing typical symptoms of canker and dieback. More details of the collected and characterized isolates are summarized in Table 2.

Phylogeny

The MP analysis of the combined dataset showed that of 3,116 characters, 464 were parsimony-informative, 239 were parsimony-uninformative, and 2,413 were constant. A total of 100 trees were retained. Tree length was equal to 1,178, CI = 0.771, RI = 0.953, and RC = 0.735. The best-fit model of nucleotide evolution based on the AIC resulted GTR + I + G for ITS, HKY + G for *tub2* and GTR + G for *tef1*-α. The ML analysis showed that of 3,116 total characters, 2,413 were constant characters and 564 were parsimony informative. Isolates AB2, AB4, AB5, AC5, AC7, AC9, AC10 and AC11 strongly clustered within the clade of *B. dothidea* (81% MP bootstrap support and 81% ML bootstrap support). Isolate AC20 strongly grouped within the clade of *Lasiodiplodia citricola,* (74/85). Isolates AC28, AC29,

Table 2. Information on fungal isolates collected and processed in this study from 11 avocado orchards. * identifies the representative isolates preliminarily identified based on the *tub2* locus. ¥ identifies the isolates fully characterized (ITS + *tub2*+ *tef1*-α) and included in the phylogenetic analyses. × indicates that the representative isolates were excluded from the phylogenetic analyses, because they were identified as *Neofusicoccum parvum* in the preliminary *tub2* locus characterization.

Orchard No.	Location (Province)	Tree age	Symptoms	Collected *Botryosphaeriaceae*	No. of representative isolates*	No. of chosen isolates¥	Species
1	Fiumefreddo (Catania)	-	Dieback	9	8	5	*N. luteum* (5), *N. parvum* (3)
2	Ramacca (Catania)	2-3 yrs	Dieback	5	5	5	*N. cryptoaustrale* (5)
3	Riposto (Catania)	2-4 yrs.	Canker	5	5	5	*B. dothidea* (5)
4	Mascali (Catania)	2-3 yrs.	Canker, dieback, grafting point canker	26	10	8	*B. dothidea* (3), *L. citricola* (1), *M. phaseolina* (4), *N. parvum* (2)
5	Agnone Bagni (Siracusa)	2-4 yrs.	Canker, dieback, grafting point canker	8	8	×	*N. parvum* (8)
6	Fiandaca (Catania)	-	Canker, dieback	5	2	×	*N. parvum* (2)
7	Acireale (Catania)	-	Dieback, grafting point canker	7	4	×	*N. parvum* (4)
8	Messina (ME)	-	Discolouration	3	2	×	*N. parvum* (2)
9	Noto (SR)	2 yrs.	Canker, dieback, grafting point canker	17	9	×	*N. parvum* (9)
10	Giarre (Catania)	Mature trees	Canker, dieback	9	1	×	*N. parvum* (1)
11	Riposto (Catania)	3 yrs.	Canker	12	8	×	*N. parvum* (8)

AC51 and AC52 grouped in the clade of *Macrophomina phaseolina* (73/81). Regarding *Neofusicoccum*, for isolates AVORAM1 to 5 the bootstrap support was 52 for the MP analysis and 60 for the ML analysis. These isolates were accommodated within *Neofusicoccum cryptoaustrale*. Isolates AVF3, AVF5, AVF6, AVF7, AVF8 were strongly supported (99/99) within the clade of *Neofusicoccum luteum*.

According to these results, five species isolated from avocado in this study were identified, including: *B. dothidea*, *L. citricola*, *M. phaseolina*, *N. cryptoaustrale*, and *N. luteum* (Figure 2). The ITS, *tub2*, and *tef1*-α sequences generated in this study were deposited in GenBank (Table 1).

Morphological and cultural characteristic of the isolates

Observing pure cultures on PDA, a total of six groups of *Botryosphaeriaceae*-like fungi were observed:

Isolate AC5 (*B. dothidea*) had olivaceous colonies that became grey with black reverse sides. Conidia were hyaline, fusiform and measured 23.2 × 5.6 µm.

Lasiodiplodia citricola AC20 had colonies with abundant aerial mycelium that became smoke grey to olivaceous-grey or iron-grey on the surfaces and greenish grey to dark slate blue on the reverse sides. Conidia were initially hyaline, aseptate, ellipsoid to ovoid and becoming pigmented, verrucose and ovoid, and measured 21.3 × 13.1 µm.

Macrophomina phaseolina isolate AC29 had grayish fluffy aerial mycelium on the colony surfaces, which were purplish grey on the reverse sides. Abundant microsclerotia were produced on pine needles in AT medium. Conidia were 25.0 × 10.5 µm.

The colonies of isolate AVORAM4 (*N. cryptoaustrale*) were initially white with fluffy aerial mycelium, changing to straw-yellow after 3 d incubation and then to pale olivaceous-grey. Conidia were hyaline, smooth with granular contents, aseptate, fusiform, and measured 20.0 × 6.0 µm.

Isolate AVF5 (*N. luteum*) was initially white with fluffy aerial mycelium and changed to yellow after 3-4 d incubation, after which the colour changed to pale olivaceous-grey from the middle of the colonies to the irregular margins. Conidia were hyaline, thin walled, aseptate, smooth, ellipsoidal, and measured 19.5 × 5.5 µm.

Isolates of *N. parvum* had white fluffy aerial mycelium that became grey and then black with the age. Conidia were hyaline, non-septate, and subglobose, with obtuse apices, and measured 18.2 × 6.1 µm.

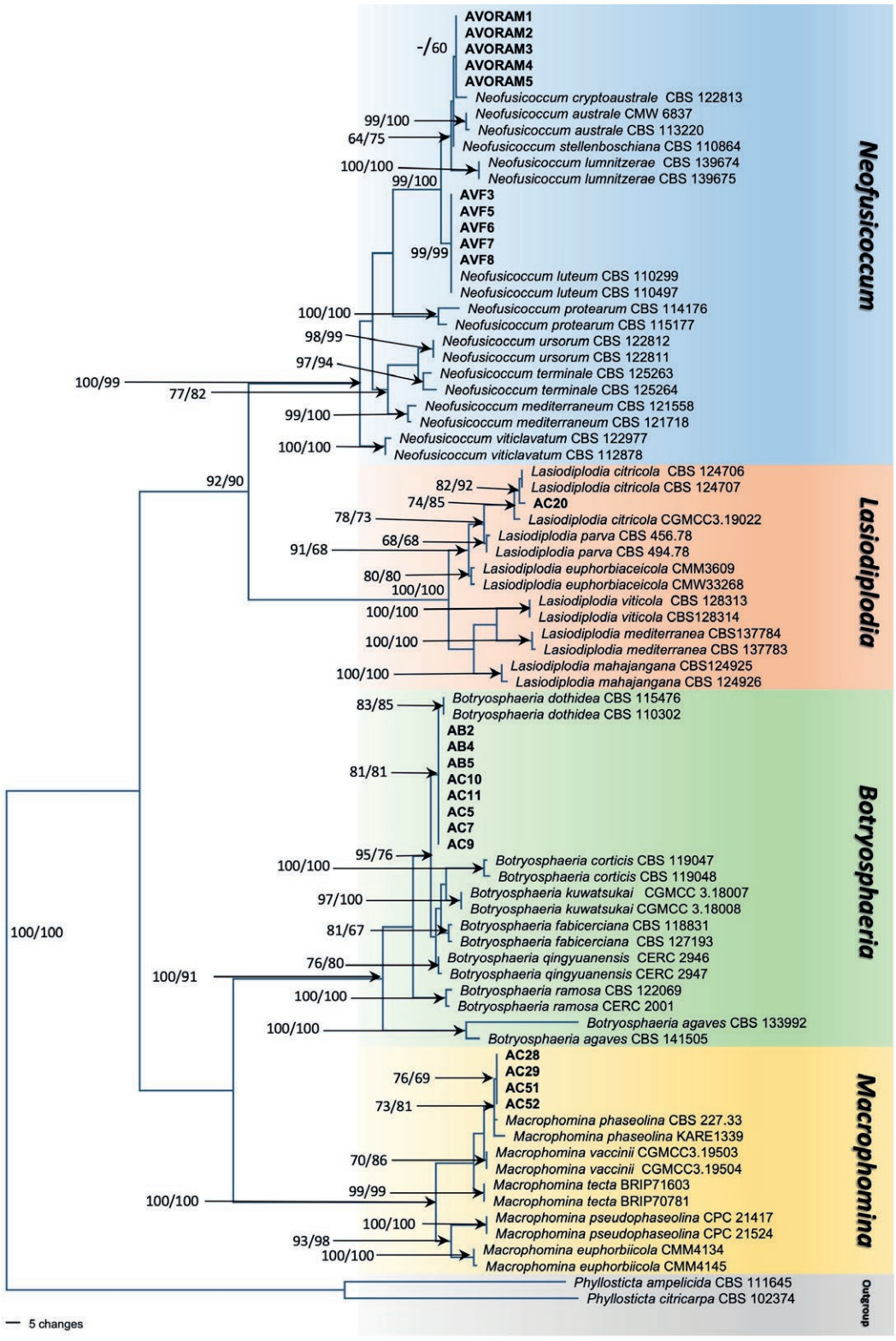

Figure 2. One of 100 equally most parsimonious trees generated from maximum parsimony analysis of three-loci (ITS + *tub2* + *tef1*-α) combined dataset of *Botryosphaeriaceae* species. Numbers before after slashes represent, respectively, parsimony and likelihood bootstrap values from 1,000 replicates. *Phyllosticta ampelicida* (CBS 111645) and *Phyllosticta citricarpa* (CBS 102374) were the outgroup taxa in both analyses. Isolates in bold font were generated in the present study. Bars indicate the numbers of nucleotide changes.

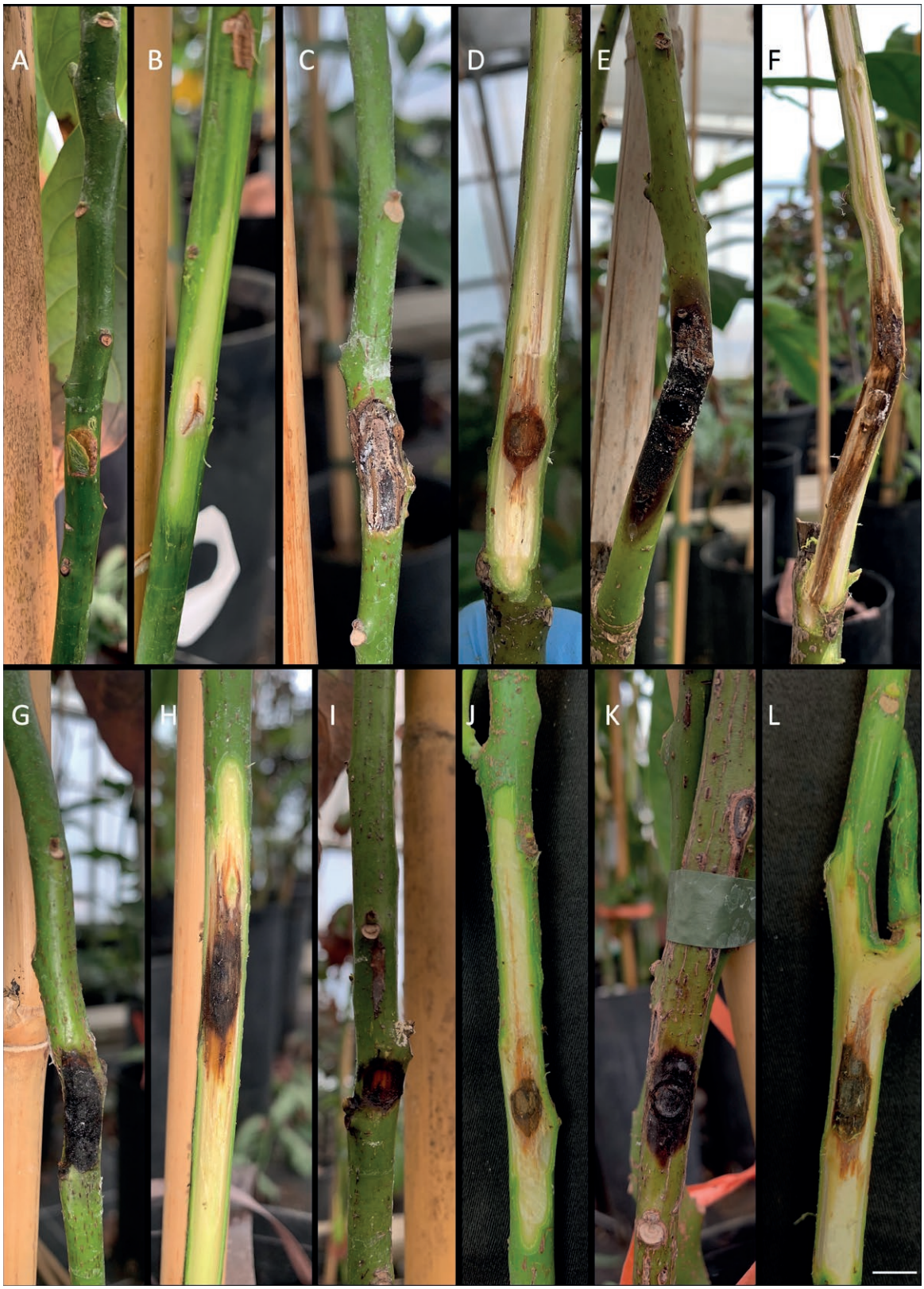

Figure 3. Result of the pathogenicity test after 63 days. **A**-**B**, Control. **C**-**D**, Shoots inoculated with *Botryosphaeria dothidea*. **E**-**F**, *Neofusicoccum luteum*. **G**-**H**, *Neofusicoccum cryptoaustrale*. **I**-**J,** *Lasiodiplodia citricola*. **K**-**L**, *Macrophomina phaseolina*. Scale bar: 2 cm.

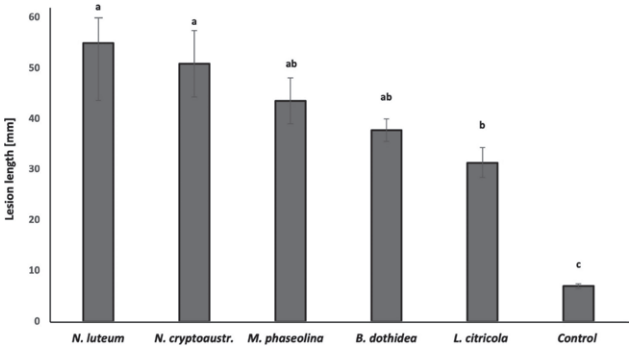

Figure 4. Mean lesion lengths (mm) resulting from the pathogenicity test of *Botryosphaeria dothidea, Lasiodiplodia citricola, Macrophomina phaseolina, Neofusicoccum cryptoaustrale,* and *N. luteum* on potted plants. Values are each for two inoculation points per plant for each fungal species. Control consisted of the same number of inoculation points. Vertical bars represent standard errors of the means. Bars accompanied with different letters indicate means that were significantly different (Fisher's protected LSD test; $\alpha = 0.05$).

Pathogenicity test

The pathogenicity showed that all the *Botryosphaeriaceae* species in this study were pathogenic to avocado plants, and produced similar symptoms to those observed in the field. All the inoculated species produced external and internal discolouration lesions. The inoculation controls did not show any symptoms (Figure 3). After 15 d, all the inoculated trees showed dark discolouration of the outer layers of bark. In detail, *N. luteum* isolate AVF5 produced the longest lesions (mean = 55.0 mm), followed by *N. cryptoaustrale* isolate AVORAM4 (50.9 mm), *M. phaseolina* isolate AC29 (43.6 mm), *B. dothidea* isolate AC7 (37.8 mm) and *L. citricola* isolate AC20 (31.4 mm). All the inoculated fungi produced lesion lengths that were statistically different from the controls ($P < 0.05$), and only lesions from *Neofusicoccum* sp. were significantly different compared to those from *L. citricola* (Figure 4). Re-isolations showed gave colonies with the morphological characteristics the same as the inoculated species, fulfilling Koch's postulates.

DISCUSSION

This study has elucidated the diversity of *Botryosphaeriaceae* species causing avocado canker and dieback in commercial orchards in Italy. The species characterized were *B. dothidea, L. citricola, M. phaseolina, N. cryptoaustrale,* and *N. luteum*. *Neofusicoccum parvum* was also constantly encountered during field surveys. This species had been characterized in a previous study (Guarnaccia *et al.* 2016), and was here characterized only on the basis of *tub2* locus, and excluded from the phylogenetic analysis. This research confirms that *N. parvum* was the predominant Botryosphaeriaceous species associated with canker and dieback symptoms of avocado in Sicilian orchards.

Botryosphaeria dothidea is the type species of *Botryosphaeria* (Marsberg *et al*., 2017), and has been reported from many plant species with broad global distribution. There are 1,260 fungus-host records for *B. dothidea* and its synonyms listed in the Fungal Database (Farr and Rossman, 2022). However, some of these reports are outdated causing taxonomic confusion. Batista *et al.* (2021) report that *B. dothidea* was associated with 403 hosts in 66 countries. McDonald and Eskalen (2011) reported fungi belonging to the *Botryosphaeriaceae*, including *B. dothidea* (*Fusicoccum aesculi*), have been associated with avocado branch cankers in California. Previous field surveys conducted in Sicily on different perennial crops, including pistachio, walnut and *Ficus* spp., recorded presence of *B. dothidea* and other *Botryosphaeriaceae* (Gusella *et al*., 2020; 2022; Fiorenza *et al*., 2022a). In the present study, within *Lasiodiplodia,* the *L. citricola* was occasionally isolated from symptomatic avocado branches, as were *M. phaseolina* and *B. dothidea*. The pathogenicity test confirmed the aggressiveness of *L. citricola* on avocado woody tissues. Different species of *Lasiodiplodia,* including *L. citricola*, have been reported to cause diseases in multiple fruit and nut tree hosts (Úrbez-Torres *et al*., 2008, 2010; Chen *et al*., 2013a, 2013b, 2013c; 2014; Carlucci *et al*., 2015; Rodríguez-Gálvez *et al*., 2017). In Sicily, *L. citricola* was recently identified as a serious threat to *Acacia* spp. causing dieback (Costanzo *et al*., 2022). On avocado, recent studies have described *L. laeliocattleyae, L. pseudotheobromae* and *L. theobromae* as etiological agents of fruit stem-end rot and dieback (Garibaldi *et al*., 2012; Qui *et al*., 2020; Rodríguez-Gálvez *et al*, 2021; Avenot *et al*., 2022).

Macrophomina phaseolina is widely distributed and is a serious threat to different crops (Baird *et al*., 2003; Sarr *et al*., 2014). This pathogen causes charcoal rot of soybean (Sarr *et al*., 2014), chickpea (Dell'Olmo *et al*., 2022), sunflower (Bokor, 2007), sorghum (Sharma *et al*., 2014), and strawberry (Koike, 2008). It has also been reported to cause diseases on woody hosts, such as grapevine (González and Tello, 2011; Nouri *et al*., 2018), olive (Sergeeva *et al*., 2005), pistachio (Nouri *et al*., 2020), and almond (Inderbitzin *et al*., 2010). *Macrophomina phaseolina* was thought to be one of the pathogens causing avocado root rot in Australia (Poudel *et al*., 2021), but it has not been recorded as causing canker on this host. Based on previous studies in Italy, on fruit and

ornamental hosts showing typical symptoms of *Botryosphaeriaceae*, including canker and dieback of woody tissues, *M. phaseolina* has not been previously isolated. This is the first report of *M. phaseolina* on avocado. Further investigations are required need to clarify the geographic extent this species in Italy, and its association with different host plants.

Neofusicoccum cryptoaustrale was detected in only one of the sampled avocado orchards. This fungus was first described Eucalyptus trees in South Africa (Crous *et al*., 2013; Pavlic-Zupanc *et al*., 2017), and was reported on ornamental and fruit crops, including *Pistacia lentiscus* (Linaldeddu *et al*., 2016), *Olea europea* (van Dyk *et al*., 2021; Hernández-Rodríguez *et al*., 2022), and mangrove species (Osorio *et al*., 2017). This fungus formed a cryptic sister species with *N. australe* (Crous *et al*., 2013). Results of the present study showed that the isolates from avocado clustered with the type isolate of *N. cryptoaustrale* (CBS 122813), close to the well supported clade of *N. australe*. We do not exclude that the present study isolates identified as *N. cryptoaustrale* could be re-accommodated following progress with multi-locus phylogeny. *Neofusicoccum luteum* is well known as a canker pathogen of avocado, and has been reported to cause branch canker and stem-end rot on avocado in California (McDonald *et al*., 2009; 2011; Twizeyimana *et al*., 2013; Avenot *et al*., 2022), Australia (Tan *et al*., 2019), New Zealand (Hartill, 1991; Hartill and Everett 2002), and Chile (Tapia *et al*., 2020). This fungus was also identified in California as the main cause of stem-end rot in harvested avocado fruit (Twizeyimana *et al*., 2013).

Despite of the diversity of *Botryosphaeriaceae* identified in the present study, *N. parvum* was the most prevalent species associated with canker and dieback of avocado, since it was detected from seven sampled locations with a high isolation frequency, as was previously reported in Italy by Guarnaccia *et al*. (2016) and in Spain by Arjona-Girona *et al*. (2019). *Neopestalotiopsis* also came from symptomatic tissues showing cankers and discolouration, but was not included in this study since it was already reported and described by Fiorenza *et al*. (2022b). Pathogenicity tests showed that representative isolates caused lesions on healthy plants. These data demonstrated that all the inoculated fungi were pathogenic to avocado, and that the isolates characterized as *N. cryptoaustrale* and *N. luteum* were the most virulent compared those of *B. dothidea, M. phaseolina*, and *L. citricola*.

Botryosphaeriaceae species have been reported as pathogens of the ornamentals to the agricultural crops in Italy, especially in Sicily (Ismail *et al*., 2013; Guarnaccia *et al*., 2016; Aiello *et al*., 2020a, 2022; Gusella *et al*., 2020, 2021, 2022; Bezerra *et al*., 2021; Fiorenza *et al*., 2022a; Costanzo *et al*., 2022). Of the studies in Italy, *Botryosphaeriaceae* have been commonly encountered in different hosts and environments. Presence of contiguous susceptible hosts and the polyphagous behaviour of this pathogen family can guarantee inoculum survival in nurseries, open fields, and urban areas. The fungi characterized in the present study have also been described on other hosts. *Botryosphaeriaceae* (including those detected in this study) are endophytes, able to induce latent infections (Slippers and Wingfield, 2007). It is possible to detect the levels of latent infections using qPCR (Luo *et al*., 2017; 2019; 2020; 2021).

The orchards investigated in the present study contained mainly young avocado trees (2 to 4-year-old). Presence of *Botryosphaeriaceae* spp. within the tissues in young trees indicates that most of the infections may originate nurseries, and then spread once the trees are transplanted in open fields. In Sicily, avocado trees are imported from other Mediterranean countries, because there are no nurseries specialized in avocado propagation. For these reasons, monitoring of latent infections, and attention during nursery propagation, are needed to avoid or limit *Botryosphaeriaceae* infections and new sources of inoculum.

This study presents updated results on the association of *Botryosphaeriaceae* species causing canker and dieback on avocado in Italy. The surveys and analyses have elucidated the diversity of this group of fungi involved in avocado canker diseases. Further studies are required to elucidate the epidemiology, control, and latent pathogenic status of *Botryosphaeriaceae* on avocado. This study is also the first to report *L. citricola*, *M. phaseolina* and *N. cryptoaustrale* causing canker and dieback on avocado trees, and is the first report of the recorded fungi causing branch disease on avocado in Italy.

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