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Woody Canker and Shoot Blight Caused by Botryosphaeriaceae and Diaporthaceae on Mango and Litchi in Italy

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Abstract: In recent years, the cultivation of tropical fruit crops has increased in the Mediterranean basin, especially in southern Italy. In surveys conducted from 2014 to 2019 woody canker and shoot blight were observed on mango plants (cvs. Kent, Keitt, Sensation, Osteen, and Kensington Pride) and litchi plants (cvs. Way Chee and Kwai Mai Pink) cultivated in Sicily. Botryosphaeriaceae and Diaporthaceae were consistently isolated from symptomatic samples. Morphological characterization and multi-locus phylogenies using three genomic loci (a portion of translation elongation factor 1-α gene, a portion of the β-tubulin gene, and an internal transcribed spacer) identified these fungi as *Neofusicoccum parvum*, *Lasiodiplodia theobromae*, *Botryosphaeria dothidea*, *Diaporthe foeniculina*, and *Diaporthe baccae* on mango and *Diaporthe foeniculina* and *Diaporthe rudis* on litchi. Pathogenicity tests on healthy mango (cv. Kensington Pride) and litchi (cv. Way Chee) plants demonstrated the pathogenicity of the isolates used in the study, and Koch's postulates were fulfilled for all pathogens. To our knowledge, this is the first report of *L. theobromae*, *B. dothidea*, and *Diaporthe* species on mango in Italy and the first report worldwide of woody canker and shoot blight caused by *D. foeniculina* and *D. rudis* on litchi plants.

Keywords: Botryosphaeriaceae and Diaporthaceae; *Mangifera indica*; *Litchi chinensis*; multi-locus phylogenies

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1. Introduction

In recent years, tropical crop cultivation has increased in the Mediterranean basin, and especially in Italy where the environmental conditions have allowed an intensification of their production. These crops represent a sector of great interest for their high nutritional value, antioxidant properties, and high income generated by the fruit market. In particular, the nutritional composition of some crops such as mango and litchi has been extensively studied for human nutrition, health, and other applications in the pharmaceutical and food industries [1–3]. Moreover, mango peels have recently been used to develop biodegradable or edible packing materials [4]. Due to their tropical origin, the cultivation of these crops is limited to southern Italy (Calabria) and insular Italy (Sardinia and Sicily). In Sicily, the cultivation of mango and litchi is mainly localized in the north-eastern coastal area of the island.

Mango (*Mangifera indica* L.) is one of the most popular fruit crops in the world and production is concentrated in Asia, Africa, America, and Oceania [5]. In Italy, the cultivation of mango for commercial purposes started during 1980–1990 in the Catania province

(eastern Sicily). Thereafter, its cultivation expanded to the other provinces of the island reaching an area of approximately 200 ha [6]. In this species, the occurrence of fungal and bacterial infections pre- and post-harvest can compromise plant growth and fruit quality. Fortunately, only a few diseases have been reported to date in Italy. Among these, woody cankers and stem-end rot of fruit caused by *Neofusicoccum* spp. and decay and stem-end rot of fruit caused by some species of *Colletotrichum* are the major diseases limiting the production of mango [7,8]. These pathogens lead to substantial crop losses, quality changes, market value decreases, and in some cases post-harvest losses, especially during ripening and storage. Moreover, leaf spots caused by *Pestalotiopsis uvicola* and *P. clavispora* [9] and wilt caused by *Verticillium dahliae* were also reported [10]. Regarding bacterial diseases, apical necrosis caused by *Pseudomonas syringae* pv. *syringae* is a major disease in the Mediterranean area and it has been described as being present in different countries including Italy [11–14].

Southeast Asia (China, Vietnam, Thailand, and India) represents the main world producer of litchi (*Litchi chinensis* Sonn.), followed by Australia, Africa, the Mediterranean basin, and tropical America [15]. Several fungal diseases affecting litchi have been reported worldwide. Downy blight caused by *Phytophthora litchii* [16] and anthracnose and litchi pepper spot caused by *Colletotrichum gloeosporioides*, *C. siamense*, and *C. simmondsii* [17,18] are considered the major pre-harvest diseases. Moreover, leaf, panicle, and fruit blights caused by *Alternaria alternata* [19] and algal leaf spots caused by *Cephaleuros virescens* were reported to cause severe economic losses. In Italy, no fungal diseases were reported on litchi.

Mango and litchi have had a great economic impact on the European market, and few studies were conducted on these crops in Italy until now. For this reason, it is necessary to investigate the diseases affecting these crops, which could represent a limiting factor for their production, in order to develop effective management strategies. In recent years with the rapid expansion of their production, some diseases were observed in mango and litchi orchards. Surveys carried out since 2014 led us to detect severe symptoms of woody canker, shoot blight, and dieback on mango and litchi plants in the main growing area of north-eastern Sicily.

The aims of this study were to (i) evaluate the etiology of the disease by morphological and molecular characterization of causal agents associated with these symptoms and (ii) determine their pathogenicity.

2. Materials and Methods

2.1. Field Survey and Isolation

Between 2014 and 2019, surveys conducted in north-eastern Sicily (Catania and Messina provinces) revealed the presence of 3–4-year-old mango plants (cvs. Kent, Keitt, Sensation, Osteen, and Kensington Pride) and 7–8-year-old litchi plants (cvs. Way Chee and Kwai Mai Pink) showing woody canker and shoot blight. Symptomatic samples were collected and brought to the laboratory of the Department of Agriculture, Food and Environment, University of Catania, for further investigations. For culture isolation, symptomatic woody tissues were surface-disinfected for 1 min in 1.2% sodium hypochlorite, rinsed in sterile water, placed on potato dextrose agar (PDA 3.9%, Oxoid, Basingstoke Hampshire, UK) amended with 100 mg/L of streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA), and then incubated at 25 ± 1 °C for three days. Subsequently, from the margin of each developing colony, a piece of mycelium was taken to obtain pure cultures. After about 5–7 days, a single conidium or hyphal tip from each pure culture was transferred to PDA and maintained at 25 ± 1 °C.

2.2. Morphological Characterization

For the morphological characterization of the pathogen, the isolates were transferred to PDA and malt extract agar (MEA; 2% malt extract, Merck, Darmstadt, Germany) supplemented with streptomycin sulfate (100 µg/mL) and incubated at 25 °C. In addition, technical agar (TA, 1.2% agar, Oxoid, Basingstoke Hampshire, UK) with sterilized pine needles placed onto the surface (PNA) and then maintained at room temperature under near-ultraviolet light (NUV) was used to encourage production of pycnidia. The size and shape of conidia and colour and shape of pycnidia/conidiomata were examined. Conidia were measured by mounting fungal structures in clear lactic acid on microscope slides and observing under an Olympus BX 61 light microscope. Fifty measurements of conidia were made for one representative isolate for each fungal species recovered.

2.3. Molecular Characterization

Mycelium was collected from 5-day-old fungal cultures grown on PDA and MEA medium. Total fungal DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). The internal transcriber spacer region (ITS) of the rDNA was amplified with primers ITS4 and ITS5 [20], the primers EF1-728F and EF1-986R [21] were used to amplify part of the translation elongation factor 1alpha gene (*tef1*), and primer sets Bt-2a and Bt-2b [22] were used for the partial betatubulin gene (*tub2*). Amplification by polymerase chain reaction (PCR) was performed in a total volume of 25 µL using OneTaq 2X Master Mix with Standard Buffer (BioLabs, Ipswich, MA, USA), according to the manufacturer's instructions on an Eppendorf AG 22331 Hamburg Mastercycler. One reaction was composed of 12.5 µL of OneTaq 2X Master Mix, 0.5 µL of each primer, and 10.5 µL of nuclease-free water. The thermal cycle consisted of an initial denaturation temperature of 94 °C for 120 s, followed by 35 cycles at the denaturation temperature of 94 °C for 30 s, the annealing temperature of 55 °C (ITS), 57–59 °C (*tef1*), or 58 °C (*tub2*) for 30 s, the elongation temperature of 72°C for 40 s (ITS), 1 min (*tef1*), or 50 s (*tub2*), and a final extension at 72 °C for 5 min. The amplification products were separated by 1% agarose gel electrophoresis, purified and sequenced by Macrogen Inc. (South Korea) using both PCR primers. Purified sequence reactions were analyzed by an Applied Biosystems 3730 × l DNA Analyzer (Life Technologies, Carlsbad, CA, USA). The generated DNA sequences were analyzed, and consensus sequences were edited using the MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms [23] and submitted to GenBank.

2.4. Phylogenetic Analysis

New sequences obtained in this study were blasted against the NCBI's GenBank nucleotide database to determine the closest sequences for a taxonomic arrangement of the studied isolates. Alignments of different gene regions, including sequences obtained from this study and sequences retrieved from GenBank, were initially performed with the MAFFT v. 7 online server (http://mafft.cbrc.jp/alignment/server/index.html, accessed on 15 February 2022) [24] and then manually adjusted in MEGA v. 7 [25]. A phylogenetic analysis was conducted as concatenated multilocus sequence analyses using the ITS, *tef1*, and *tub2* combination for the *Botryosphaeria*, *Diaporthe*, *Lasiodiplodia*, and *Neofusicoccum* genera [26,27]. The ex-type strain of *Neofusicoccum parvum* (CMW9081) was used as an outgroup for the analysis of *Botryosphaeria* spp., *Diaporthella corylina* (CBS 121124) was used as an outgroup for *Diaporthe* spp., *Neodeightonia phoenicum* (CBS 122528) was used as an outgroup for *Lasiodiplodia* spp., and *Botryosphaeria dothidea* (CBS 110302) was used as an outgroup for *Neofusicoccum* spp. The phylogenies were based on Bayesian Inference (BI) for the multi-locus analyses. The best evolutionary model was determined for each partition using MrModeltest v. 2.3 [28] and incorporated into the analyses. MrBayes v. 3.2.5 [29] was used to generate dendrograms under optimal criteria per partition. The Markov Chain Monte Carlo (MCMC) analysis used four chains and started from a random tree topology. The heating parameter was set at 0.2 and trees were sampled every 1000 generations.

Analyses stopped when the average standard deviation of split frequencies was below 0.01. Sequences generated in this study were deposited in GenBank (Table 1).

Table 1. Fungal isolates collected in this study and their Genbank accession numbers.

Fungal Species	Culture No.	Host/Cultivar	Genbank Accession Number		
			ITS	*tef1*	*tub2*
Lasiodiplodia theobromae	KEN1	*Mangifera indica* cv. Kent	OL470874	OL891701	OL891738
L. theobromae	KEN2	*M. indica* cv. Kent	OL470875	OL891699	OL891742
L. theobromae	KEN3	*M. indica* cv. Kent	OL470876	OL891700	OL891739
L. theobromae	KEN4	*M. indica* cv. Kent	OL470877	OL891708	OL891741
L. theobromae	KEN5	*M. indica* cv. Kent	OL470878	OL891709	OL891740
Neofusicoccum parvum	KEI1	*M. indica* cv. Keitt	OL470866	OL891684	OL891757
N. parvum	KEI2	*M. indica* cv. Keitt	OL470867	OL891690	OL891756
N. parvum	KEI3	*M. indica* cv. Keitt	OL470868	OL891695	OL955497
N. parvum	KEI4	*M. indica* cv. Keitt	OL470869	OL891696	OL891751
N. parvum	KEI5	*M. indica* cv. Keitt	OL470870	OL891697	OL891752
N. parvum	KEI6	*M. indica* cv. Keitt	OL470871	OL891698	OL891753
N. parvum	KEI7	*M. indica* cv. Keitt	OL470872	OL891710	OL891754
N. parvum	KEI8	*M. indica* cv. Keitt	OL470873	OL891711	OL891759
N. parvum	SENS1	*M. indica* cv. Sensation	OL470882	OL891688	OL955498
N. parvum	SENS2	*M. indica* cv. Sensation	OL470883	OL891689	OL955500
N. parvum	SENS3	*M. indica* cv. Sensation	OL470884	OL891713	OL891750
N. parvum	SENS4	*M. indica* cv. Sensation	OL470885	OL891691	OL955496
N. parvum	SENS5	*M. indica* cv. Sensation	OL470886	OL891692	OL891758
N. parvum	SENS6	*M. indica* cv. Sensation	OL470887	OL891712	OL955495
N. parvum	SENS7	*M. indica* cv. Sensation	OL470888	OL891693	OL955499
N. parvum	SENS8	*M. indica* cv. Sensation	OL470889	OL891694	OL955494
N. parvum	OSTB1	*M. indica* cv. Osteen	OL470879	OL891685	OL891749
N. parvum	OSTB2	*M. indica* cv. Osteen	OL470880	OL891686	OL891760
N. parvum	OSTB3	*M. indica* cv. Osteen	OL470881	OL891687	OL891755
Diaporthe foeniculina	OSTD1	*M. indica* cv. Osteen	OL477409	OL891674	OL891729
D. foeniculina	OSTD2	*M. indica* cv. Osteen	OL477410	OL891675	OL891730
D. foeniculina	OSTD3	*M. indica* cv. Osteen	OL477411	OL891682	OL891731
D. foeniculina	OSTD4	*M. indica* cv. Osteen	OL477412	OL891676	OL891720
Botryosphaeria dothidea	MG2	*M. indica* cv. Kensington Pride	OL470890	OL891704	OL891746
B. dothidea	MG3	*M. indica* cv. Kensington Pride	OL470891	OL891706	OL891743
B. dothidea	MG4	*M. indica* cv. Kensington Pride	OL470892	OL891702	OL891748
B. dothidea	MG5	*M. indica* cv. Kensington Pride	OL470893	OL891705	OL891744
B. dothidea	MG6	*M. indica* cv. Kensington Pride	OL470894	OL891707	OL891745
B. dothidea	MG7	*M. indica* cv. Kensington Pride	OL470895	OL891703	OL891747
D. foeniculina	MG9	*M. indica* cv. Kensington Pride	OL477413	OL891678	OL891722
D. baccae	MG11	*M. indica* cv. Kensington Pride	OL477414	OL891681	OL891733
D. foeniculina	MG13	*M. indica* cv. Kensington Pride	OL477415	OL891677	OL891727
D. foeniculina	MG14	*M. indica* cv. Kensington Pride	OL477416	OL891679	OL891728
D. foeniculina	MG15	*M. indica* cv. Kensington Pride	OL477417	OL891683	OL891721
D. foeniculina	LC1	*Litchi chinensis* cv. Way Chee	OL477395	OL891664	OL891714
D. foeniculina	LC2	*L. chinensis* cv. Way Chee	OL477402	OL891670	OL891723
D. rudis	LC3	*L. chinensis* cv. Way Chee	OL477403	OL891660	OL891737
D. rudis	LC4	*L. chinensis* cv. Way Chee	OL477404	OL891662	OL891734
D. rudis	LC5	*L. chinensis* cv. Way Chee	OL477405	OL891663	OL891736
D. rudis	LC6	*L. chinensis* cv. Way Chee	OL477406	OL891661	OL891735

Table 1. *Cont.*

Fungal Species	Culture No.	Host/Cultivar	Genbank Accession Number		
			ITS	*tef1*	*tub2*
D. foeniculina	LC7	*L. chinensis* cv. Way Chee	OL808652	OL891671	OL891724
D. foeniculina	LC8	*L. chinensis* cv. Way Chee	OL477407	OL891672	OL891725
D. foeniculina	LC9	*L. chinensis* cv. Kwai Mai Pink	OL477408	OL891673	OL891719
D. foeniculina	LC10	*L. chinensis* cv. Kwai Mai Pink	OL477396	OL891665	OL891732
D. foeniculina	LC11	*L. chinensis* cv. Kwai Mai Pink	OL477397	OL891666	OL891726
D. foeniculina	LC12	*L. chinensis* cv. Kwai Mai Pink	OL477398	OL891667	OL891715
D. foeniculina	LC13	*L. chinensis* cv. Kwai Mai Pink	OL477399	OL891668	OL891716
D. foeniculina	LC14	*L. chinensis* cv. Kwai Mai Pink	OL477400	OL891680	OL891717
D. foeniculina	LC15	*L. chinensis* cv. Kwai Mai Pink	OL477401	OL891669	OL891718

2.5. Pathogenicity Tests

Pathogenicity tests were conducted in controlled conditions with one representative isolate of each fungal type isolated from mango and litchi plants, except for *N. parvum*. For each isolate, six potted plants of mango and litchi (2 years old, cvs. Kengsinton Pride and Way Chee, respectively) were wounded at 3 different points on the stem. Inoculations were conducted after removing bark portions with a 5 mm diam. cork borer, placing a 5 mm plug from a 10-day-old PDA culture of the test isolate into the wound, and covering with Parafilm® (Pechney Plastic Packaging Inc., Chicago, IL, USA) to prevent desiccation and contamination. An equal number of plants were inoculated in the same way using sterile PDA plugs for control. Inoculated plants were then incubated at 25 ± 1 °C and 95% relative humidity (RH) in an 18 h fluorescent light/16 h dark regime in a growth chamber. Symptom development was evaluated after 2 months. In order to fulfill Koch's postulates, re-isolations were performed following the procedure described above. Then, each fungus obtained was identified through morphological and molecular characteristics.

3. Results

3.1. Field Survey and Morphological Characterization

Symptomatic mango plants (cvs. Kent, Keitt, Sensation, Osteen, and Kensington Pride) were observed in seven orchards (from Giarre to Fiumefreddo municipalities, Catania province), whereas litchi symptoms were observed in only one orchard in Caronia municipality (Messina province). On mango plants, disease incidence (DI) varied from 3% to 18% according to the plant cultivar and orchard investigated, with the lowest values for Kensington Pride and the highest values for Osteen. On litchi, the DI was approximately 30% for Way Chee and 1% for Kwai Mai Pink.

Field disease symptoms of mango and litchi plants are shown in Figures 1 and 2. In detail, all investigated cultivars of mango showed various external disease symptoms including shoot and twig blight, branch dieback, woody canker, and cracking of the bark. Occasionally on mango, woody canker can completely girdle the rootstock Gomera-3 and kill the tree in one to several months. Internal observation of symptomatic tissues revealed a brown wood discoloration (Figure 1). Litchi plants (cvs. Way Chee and Kwai Mai Pink) showed woody canker and shoot and twig blight. Affected leaves showed a brownish color and wilting (Figure 2).

Different types of colonies were consistently obtained from symptomatic tissues. A total of 54 fungal isolates were established from single conidium or hyphal tip cultures on the PDA (Table 1). Thirty-five isolates were characterized by dark green to gray, fast-growing mycelium on the MEA. Moreover, the isolates produced pycnidia containing pigmented or hyaline conidia on the PNA within 4 weeks. These characteristics led us to identify the fungal isolates as *Botryosphaeriaceae* spp. based on the earlier family description [30]. Fifteen isolates were characterized by slow-growing colonies on the PDA, with white, sparse, aerial mycelium and greenish-yellow pigmentation developing on reverse from the center. These isolates produced pycnidia containing hyaline conidia on

the PNA within 4 weeks. Based on these characteristics, the fungal isolates were identified as *Diaporthe* spp. [31]. The morphological characteristics of each species recognized are reported in Table 2.

Figure 1. Symptoms of woody canker and shoot blight on mango plants. (**a**–**c**) Plant death as a consequence of trunk canker on rootstock Gomera-3; (**d**) branch dieback; (**e**–**g**) different types of canker lesions; (**h**) internal discoloration under canker lesion.

Figure 2. Symptoms of woody canker and shoot blight on litchi plants. (**a**) Twig blight and defoliation; (**b**) shoot blight; (**c**,**d**) leaf blight.

Table 2. Morphological characteristics of isolates collected in this study.

Species	Morphological Characteristics			
	Colony on PDA	**Pycnidia**	**Conidia**	
Diaporthe baccae	Cream colour becoming smoky gray, flattened, dense mycelium; grayish sepia on the lower surface	Brown to black, globose with yellowish masses on MEA	α conidia aseptate, hyaline, fusiform to ellipsoidal tapering toward both ends; 5.1–9.4 × 2–9.4 µm	β conidia aseptate, hyaline, apex acutely rounded, base truncate; 16.8–27.1 × 0.8–2.2 µm
D. foeniculina	White, sparse, cottony, aerial mycelium; greenish yellow on the lower surface	Black, globose to subglobose with yellowish conidial masses on PNA	α conidia aseptate, hyaline, ellipsoidal to fusiform, rarely with substruncate base; 3.9–11.1 × 1.5–3.4 µm	β conidia aseptate, hyaline, slightly curved with substruncate base; 15.8–27.1 × 0.8–2.2 µm
D. rudis	White, fluffy, aerial mycelium; whitish yellow on the lower surface	not observed	α conidia not observed	β conidia not observed
Botryosphaeria dothidea	Olivaceous becoming grayish-dark brown, dense mycelium; grayish-dark brown on the lower surface	Black to brown, globose to subglobose on PNA	Aseptate, hyaline, fusiform to subclavate with substruncate to blunty rounded base; 17.7–27.8 × 4.2–7.4 µm	-
Lasiodiplodia theobromae	White, dense aerial mycelium becoming grayish green; yellowish gray on the lower surface	Black, globose to subglobose on PNA	Aseptate, hyaline, subovoid to ellipsoid immature, become 1-septate with age, dark brown, thick walled, ellipsoidal; 22.1–26.6 × 12.9–14.5 µm	-
Neofusicoccum parvum	White, dense aerial mycelium becoming greenish gray; greenish gray on the lower surface	Dark to brown, globose to subglobose on PNA	Aseptate, old conidia becoming 1–2 septate, hyaline, ellipsoidal with apex round and base flat; 13.3–20.9 × 5.2–7.5 µm	-

3.2. Molecular Characterization and Phylogenetic Analysis

The combined locus phylogeny of *Botryosphaeria* consisted of 21 sequences, *Diaporthe* consisted of 52 sequences, *Lasiodiplodia* 37 sequences, and *Neofusicoccum* 37 sequences including outgroups. A total of 1433 characters (ITS: 1–588, *tef1*: 591–976, and *tub2*: 980–1433) were included in the *Botryosphaeria* phylogenetic analyses. A total of 1412 characters (ITS: 1–397, *tef1*: 404–935, and *tub2*: 942–1412) were included in the *Diaporthe* phylogenetic analyses. The analyses for the *Lasiodiplodia* group consisted of 1094 nucleotides (ITS: 1–472, *tef1*: 479–758, and *tub2*: 765–1094) and the analyses of *Neofusicoccum* were based on a total of 1153 characters (ITS: 1–499, *tef1*: 506–890, and *tub2*: 897–1153). For both the Bayesian analyses, MrModeltest recommended the models SYM (symmetrical model) + I (proportion of invariable sites) + G (gamma distribution) for ITS, and GTR (generalized time-reversible model) + G for *tef1* and *tub2*. Unique site patterns for each partition and all the parameters of the Bayesian analyses are reported in Table 3. In the *Botryosphaeria* species analysis, six isolates from symptomatic mango plants clustered with the ex-type and one reference strain of *B. dothidea* (Figure 3), whereas the final tree generated for *Diaporthe* showed that four isolates from litchi grouped with two reference strains of *D. rudis*, two from mango and three strains from litchi with the ex-type strain of *D. baccae*, and seven from mango and eight strains from litchi with the ex-type strain of *D. foeniculina* (Figure 4). Five isolates

from mango clustered with the ex-type and one reference strain of *L. theobromae* (Figure 5), and 19 isolates clustered as *N. parvum* in the phylogenetic tree generated from the analysis of the *Neofusicoccum* genus (Figure 6).

Table 3. Bayesian inference characteristics of the analyses conducted in this study.

	Botryosphaeria	*Diaporthe*	*Lasiodiplodia*	*Neofusicoccum*
Locus(i)	**ITS + *tef1* + *tub2***	**ITS + *tef1* + *tub2***	**ITS + *tef1* + *tub2***	**ITS + *tef1* + *tub2***
Unique site patterns of ITS	92	318	56	44
Unique site patterns of *tef1*	114	151	108	31
Unique site patterns of *tub2*	75	241	37	39
Generation ran	845,000	1,790,000	880,000	3,195,000
Generated trees	1692	3582	1762	6392
Sampled trees	1270	2688	1322	4794

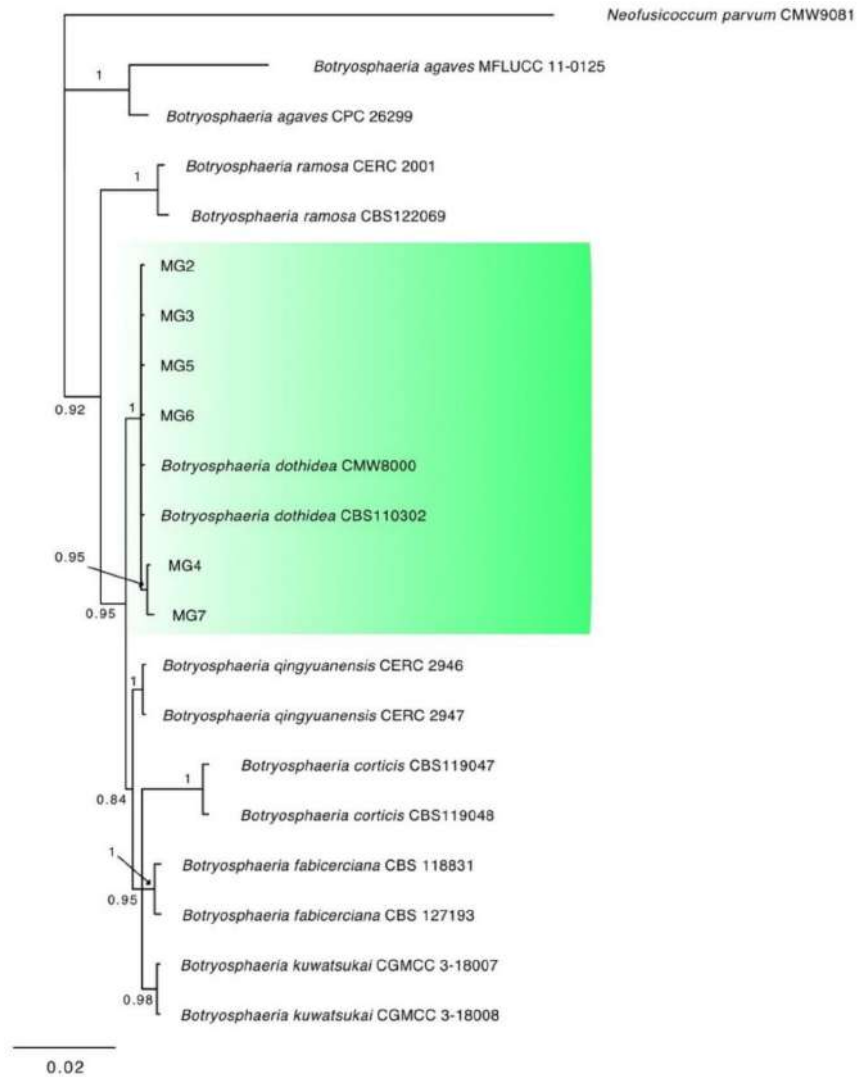

Figure 3. Consensus phylogram of 1692 trees resulting from a Bayesian analysis of the combined ITS, *tef1*, and *tub2* sequence of *Botryosphaeria* species. The tree was rooted to *Neofusicoccum parvum* (CMW9081). Bayesian posterior probability values are indicated at the nodes. The cluster containing the isolates obtained in this study is highlighted in green.

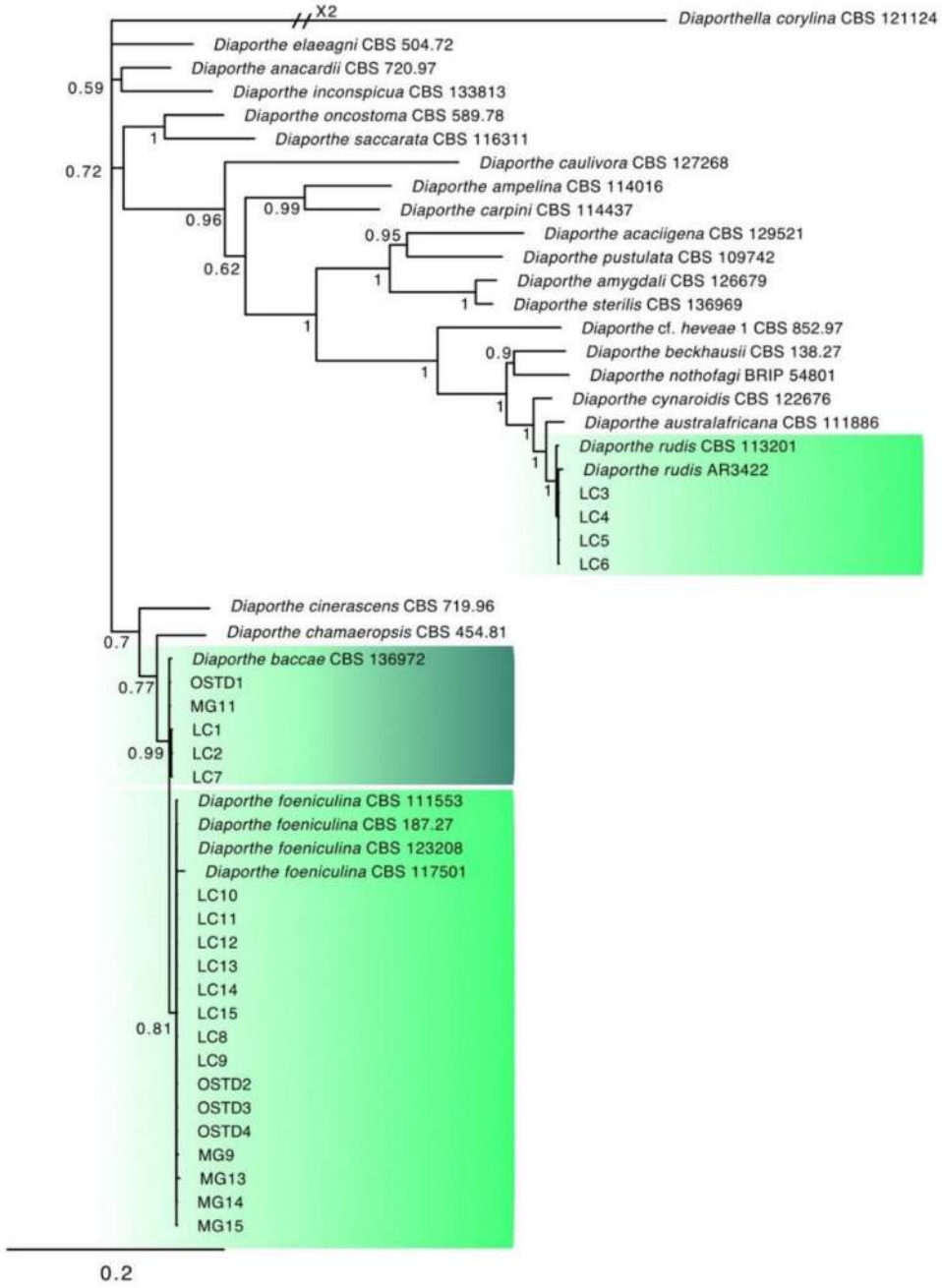

Figure 4. Consensus phylogram of 3582 trees resulting from a Bayesian analysis of the combined ITS, *tef1*, and *tub2* sequence of *Diaporthe* species. The tree was rooted to *Diaporthella corylina* (CBS121124). Bayesian posterior probability values are indicated at the nodes. The clusters containing the isolates obtained in this study are highlighted in green.

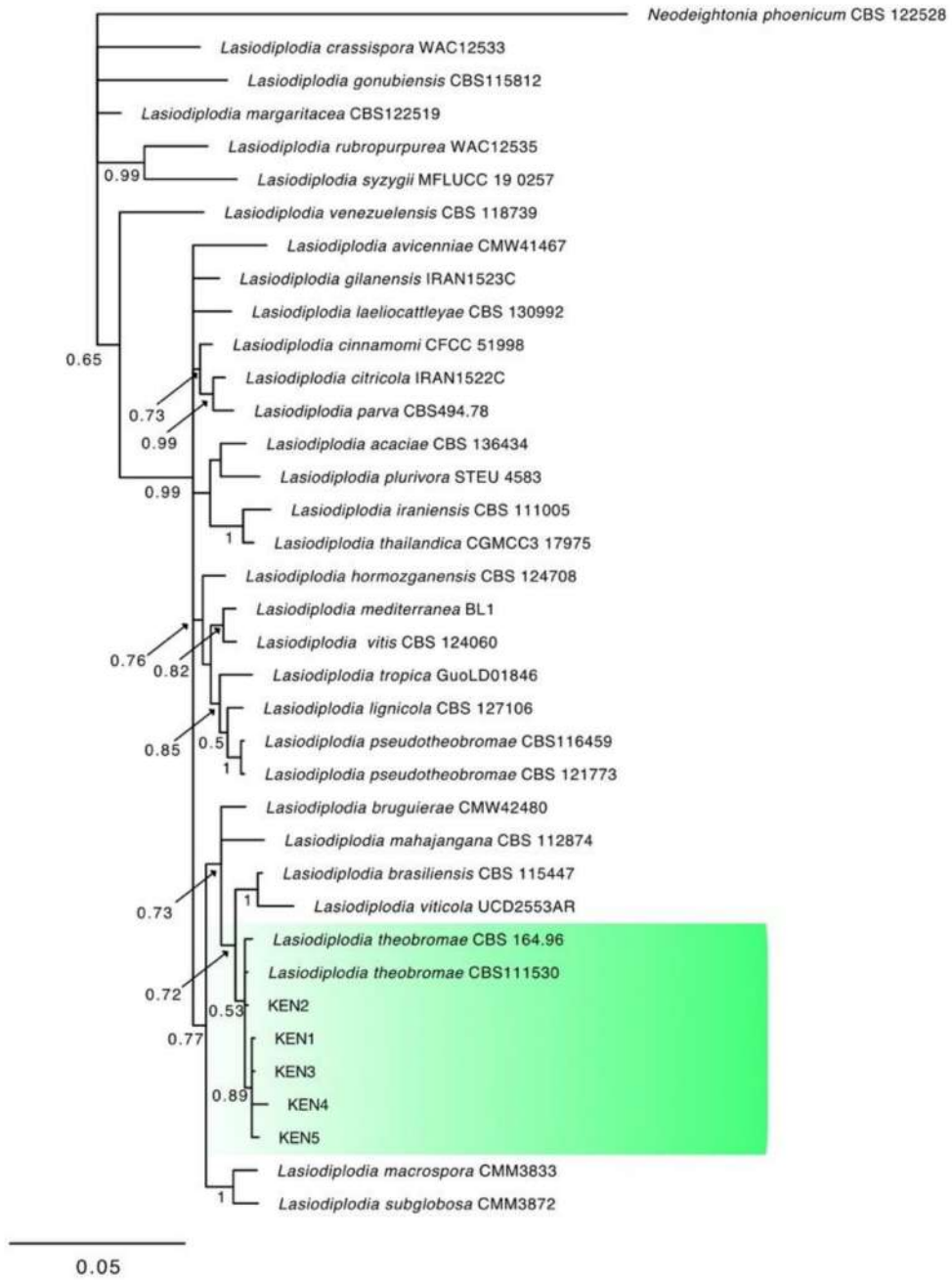

Figure 5. Consensus phylogram of 1762 trees resulting from a Bayesian analysis of the combined ITS, *tef1*, and *tub2* sequence of *Lasiodiplodia* species. The tree was rooted to *Neodeightonia phoenicum* (CBS122528). Bayesian posterior probability values are indicated at the nodes. The cluster containing the isolates obtained in this study is highlighted in green.

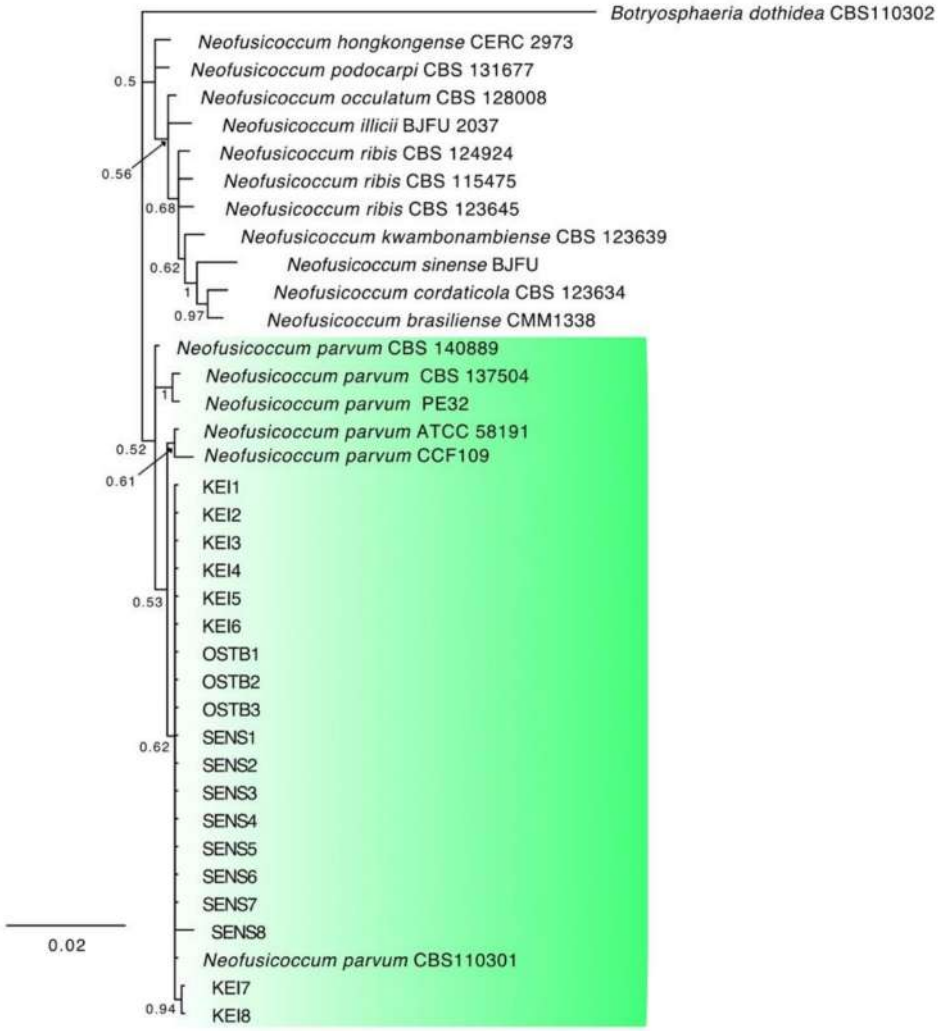

Figure 6. Consensus phylogram of 6392 trees resulting from a Bayesian analysis of the combined ITS, *tef1*, and *tub2* sequence of *Neofusicoccum* species. The tree was rooted to *Botryosphaeria dothidea* (CBS110302). Bayesian posterior probability values are indicated at the nodes. The cluster containing the isolates obtained in this study is highlighted in green.

3.3. Pathogenicity Tests

After 1 month, all the isolates of the different fungal species caused necrotic lesions on the wood of the inoculated plants. In detail, cankers and internal discoloration were observed in agreement with the inoculation points (Figures 7 and 8). All the isolates induced similar lesions on the inoculated host species and no difference in length of the resulting lesions was observed among the inoculated isolates after 1 month. Otherwise, after 3 months infection resulted in apical dieback of the mango plants when inoculated with a *D. baccae* isolate. The other isolates killed the plants after more than 6 months. Control plants showed no symptoms. The fungi were re-isolated from some of the artificially inoculated plants in order to fulfill Koch's postulates.

Figure 7. Internal discoloration of woody cankers in mango plants cv. Kensington Pride 30 days after inoculation with (**a**) *Botryosphaeria dothidea*; (**b**) *Lasiodiplodia theobromae*; (**c**) *Diaporthe foeniculina*; (**d**) *D. baccae*.

Figure 8. Woody cankers on litchi plants cv. Way Chee 30 days after inoculation with (**a**,**b**) *Diaporthe rudis*; (**c**) *D. baccae*.

4. Discussion

This study represents the first comprehensive survey of fungi associated with woody canker and shoot blight of mango and litchi plants in Sicily (southern Italy). Several cultivars of mango such as Kent, Keitt, Sensation, Osteen, and Kensington Pride from seven orchards and Way Chee and Kwai Mai Pink litchi from one orchard were inspected and sampled. Five different fungal species belonging to Botryosphaeriaceae and two species of Diaporthaceae were associated consistently with canker and dieback symptoms. Fifty-six fungal isolates were identified by morphological characterization and multi-locus phylogenies using ITS, *tef1*, and *tub2*. The discovered species included *N. parvum*, *B. dothidea*, *L. theobromae*, *D. baccae*, and *D. foeniculina* on mango and *D. rudis* and *D. foeniculina* on litchi. The pathogenicity of all Botryosphaeriaceae and Diaporthaceae species found for the first time in this study was confirmed through controlled inoculation experiments on mango and litchi plants. All isolates tested were able to cause cankers and necrotic lesions showing similar pathogenic behavior. Only *D. baccae* proved to be more aggressive killing the inoculated plants of mango after 3 months. To our knowledge, *B. dothidea*, *L. theobromae*,

D. baccae, and *D. foeniculina* as well as *D. rudis* and *D. foeniculina* are reported here for the first time on mango and litchi plants in Italy.

Diaporthaceae and Botriophaeriaceae were already present in Italy and they were reported on other host species. *Botryosphaeria dothidea* has occurred on different species such as sycamore, red oak and English oak [32], grapevine [33,34], red eucalyptus in Sardinia [35], pistachio and *Ficus microcarpa* in Sicily [36,37]; *D. baccae*, *D. rudis*, and *N. parvum* have been reported on several *Citrus* spp. and on *Vaccinum corymbosum* [38–40]; *D. foeniculina* and *N. parvum* have also been reported on avocado and citrus [39,41,42]; *L. theobromae* was reported on grapevine and avocado [43,44]; *N. parvum* was also reported affecting *Ficus carica* [45], and *D. rudis* on grapes [46].

Some Botryophaeriaceae species obtained in this study, including *B. dothidea* and *L. theobromae* were reported as causal agents of fruit rot and the decline of mango in Florida, Brazil, and Australia [47–52]. Otherwise, dieback, decline, and stem-end rot of fruit caused by *N. parvum*, the main species found in this study, were already reported in Italy [7,41]. Botryosphaeriaceae include endophytes that infect the healthy tissues of woody plants and remain dormant until the occurrence of stress conditions [52–55]. Among these, environmental conditions such as temperature, rainfall, and wind could be involved in the development of infections increasing the stress on plants. Some studies have reported that climatic change affects the pathogen, the host, and the interaction between them [56,57]. Concerning Botryosphaeriaceae, *B. dothidea* was reported as a causal agent of serious infections on weak, stressed, or off-site trees [58]. Increased incidence and symptom severity of *B. dothidea* on apple trees have been related to water stress and winter injury in the eastern USA [59]. The symptom severity of diplodia shoot blight, caused by *Diplodia sapinea*, has consistently been associated with water stress [60–62].

Diaporthe species include endophytes associated with hosts present in temperate and tropical regions [63] as well as opportunistic plant pathogens. On mango, *D. arecae*, *D. perseae*, and *D. pascoei* have been reported in Malaysia, *D. ueckerae* and *D. pseudomangiferae* have been reported in Puerto Rico and other two species in Japan [64–66]. Several *Diaporthe* species are well-established in Europe and cause diseases of different crops [38,39,41,67]. Nevertheless, this study represents the first record of woody canker and shoot blight caused by *D. baccae* and *D. foeniculina* on mango. On litchi, *Botryosphaeria* sp., *Diplodia* sp., and *Phomopis* sp. were reported as causal agents of stem canker and dieback in Florida [68], but this study represents the first report worldwide of *D. foeniculina* and *D. rudis* on this host.

The occurrence of these infections in the field compromises plant growth as well as fruit quality. Indeed, Diaporthaceae and Botryosphaeriaceae have also been reported as causal agents of fruit stem-end rot, one of the most important diseases of fruit [41,50,64,66]. Therefore, these pathogens could also be a severe limiting factor for the production and export of fruit. Moreover, the pathogens' recovery in this study confirms their high prevalence in Italy and the high infection risk for additional susceptible crops.

Since Sicily is not self-sufficient for the production of seedlings, young plants are imported from abroad, so the introduction of these pathogens in our areas could be due to the movement of infected plants from other countries. Moreover, the contamination of the material could occur during any of the production steps. For this reason, the use of certified propagative material and the elimination of dead wood or pruning residues are necessary to reduce potential inoculum sources, as well as following hygienic practices in order to avoid the inoculum spread.

Since these fungal infections could represent a major threat to the emerging tropical fruit crop cultivation in Sicily, it is necessary to conduct further research aimed at identifying the main stress factors influencing disease development and the potential damage to fruit, and to develop sustainable and effective strategies for disease management.

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