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Article

Colletotrichum perseae and *Colletotrichum gloeosporioides sensu strictu* Causing Stem Lesion and Dieback in Avocado in Italy

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Abstract

In the last decade, avocado production has increased in Italy due to the fruit's high nutritional quality and economic value. During 2024, stem lesions, wood discoloration and dieback, often starting at the grafting point, were observed in young plants in a nursery in Sicily (Italy). *Colletotrichum*-like colonies were frequently isolated from symptomatic tissues. Multi-locus phylogenetic analysis (*gapdh*, *chs-1*, *act*, *tub2*, *cal*, *gs* and ApMat) was conducted on 11 representative isolates, identifying 6 as *C. perseae* and 5 as *C. gloeosporioides sensu stricto* (*s.s.*). Two representative isolates were selected for pathogenicity tests performed on 2-year-old avocado plants cultivated in a greenhouse. After two months, necrotic lesions, wood discoloration and reddish-brown streaking at the inoculation point were induced in both species. Additional inoculations of avocado fruit confirmed the ability of both species to cause fruit rot. All inoculated fungi were successfully re-isolated and identified, fulfilling Koch's postulates. This is the first report of stem lesions and dieback caused by *Colletotrichum* species and the first occurrence of *C. perseae* in avocado plants in Europe. The results highlight the importance of early monitoring in nurseries during the propagation process and contribute to a better understanding of fungal diseases in avocado crops in Italy.

Keywords: *Persea americana*; *Colletotrichum* species; stem and fruit infections

1. Introduction

Fungi of the genus *Colletotrichum* are included among the 10 most important plant pathogenic fungi worldwide [1]. Their importance is due to the high spread and economic impact of diseases that affect a wide range of important tropical, subtropical and temperate crops. These fungi cause diseases known as anthracnose on leaves and fruit, as well as shoot and twig dieback and stem canker [2–6].

In avocado (*Persea americana*), fruit rot and stem-end rot are well known in postharvest conditions and result in internal decay and substantial economic losses. Fruit infections are mainly caused by *Colletotrichum gloeosporioides sensu stricto* (*s.s.*) [7,8], although other species from different complexes (i.e., *gloeosporioides*, *acutatum*, *boninense*, *gigasporum*, *dematium* and *magnum*) have been reported as causal agents [3,9–17]. On immature fruit, these fungi establish latent infections before harvest and symptoms occur during ripening and

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postharvest storage [18]. However, detecting fruit symptoms can be challenging in dark-skin cultivars such as 'Hass'.

Although the literature regarding avocado fruit infections caused by *Colletotrichum* spp. is extensive due to their postharvest relevance [3,7,10,14,16,17], their role in epigean tissue infections has been less explored. Dark and dry necrotic lesions on twigs and shoots affecting young avocado plants, as well as leaf spots, blossom blight and shoot blight, have been reported in Australia [19]. In addition, *Colletotrichum* species were recovered from lesions on twigs, branches or trunks of avocado in association with further fungal pathogens. For instance, Avocado Branch Canker (formerly Dothiorella Canker) in California is caused by a complex of fungal pathogens, including *Colletotrichum* spp. and multiple species in the Botryosphaeriaceae family and *Diaporthe* genus. The results of these studies suggested that *Colletotrichum* species may act as secondary invaders of avocado wood tissues [20–22].

Colletotrichum species penetrate and establish primary infections through wounds on the bark caused by mechanical injuries, frost or wind damage, and sunburn during hot summers. Pruning wounds also represent entry points for fungal spores. During winter, these pathogens survive in diseased tissues and infected debris and subsequently produce conidia that infect young tissues developing symptomatic or latent infections [18]. In this context, it is important to note that the same *Colletotrichum* isolates affecting branches or other wood tissues of avocado plants in orchards were also able to cause postharvest fruit decay [12,23,24].

In nurseries, spores of these fungi on infected tissue are spread by water splashing and infect host plants through wounds or infected propagation materials [25]. Seeds, cuttings and scions used for plant propagation may represent a source of spreading for these pathogens, leading to seed decay, stem lesions and death of the graft union. Consequently, plants may die during the nursery production process. A study carried out in Colombian avocado nurseries demonstrated *Colletotrichum gloeosporioides sensu lato* (*s.l.*) to be the causal agent of necrosis in plumules, radicles and flowers; stem rot at the grafting point; black necrotic foliar spots; branch dieback; and necrotic lesion of the stem [26].

In Italy, avocado production has increased in recent years due to the fruit's high nutritional quality and economic value, reaching more than 1000 hectares cultivated, mainly concentrated in the southern regions [27]. However, production has been affected by several fungal pathogens causing branch, trunk and shoot infections in avocado, with a higher incidence observed in young plants [12,28–30]. Extensive reports have described the presence of *Neofusicoccum parvum*, Diaporthaceae, *Neocosmospora perseae*, *Neopestalotiopsis siciliana* and *Neop. rosae* [12,29,31]. More recently, different fungi belonging to the Botryosphaeriaceae (e.g., *Botryosphaeria dothidea*, *Lasiodiplodia citricola*, *Macrophomina phaseolina*, *Neof. cryptoaustrale* and *Neof. luteum*) were found to cause necrotic lesions, canker and dieback [32]. Symptoms observed in the first months after transplanting may result from infections that occur in the nursery during the propagation process [30–33].

To date, the diversity of *Colletotrichum* species causing diseases in avocado plants in Italy has been poorly studied. Previous studies reported *C. gloeosporioides* and *C. fructicola sensu stricto* (*s.s.*) in association with Botryosphaeriaceae and *Diaporthe* spp. [12] and *Colletotrichum fioriniae* causing stem-end rot in fruit [11].

Recently, new infections affecting the shoots and stems of young avocado plants were observed in a nursery (eastern Sicily, southern Italy). In detail, the aims of this study were (i) to assess and identify, through molecular and phylogenetic analyses, the species associated with symptomatic tissues of avocado cv. Hass in the main area of production in eastern Sicily; (ii) to assess the ability of fungal species recovered to cause symptoms on stems in nursery plants; and (iii) to evaluate the pathogenicity of these fungi in avocado fruit.

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2. Materials and Methods

2.1. Field Sampling and Fungal Isolation

Between April and May 2024, a total of twenty thousand plants were surveyed in a nursery located in Lentini (CT) (37°21′50.9″ N, 15°03′41.1″ E) in the eastern area of Sicily (southern Italy). Young plants cv. Hass on 'Day' (VC 207) rootstocks showed stem lesions, wood discoloration and dieback, often starting at the grafting point and causing the death of the plants. The plants were reproduced by seed and then grafted in the nursery. Representative number of 40 symptomatic plants with visible necrotic lesions were transferred and then processed at the Laboratory of the Department of Agriculture, Food and Environment (Di3A), University of Catania.

To identify the causal agents, fungal isolations were performed from symptomatic tissues. A total of 400 small woody fragments (approximately 0.5 cm^2) taken from the margin of symptomatic tissues were surface-sterilized for one minute in a 1.5% sodium hypochlorite (NaOCl) solution, rinsed in sterile distilled water (SDW), dried on sterile paper and then placed on Petri dishes containing potato dextrose agar (PDA; Lickson, Vicari, Italy) supplemented with 100 mg L^{-1} streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA) to prevent bacterial growth. Plates were incubated in the dark at 25 ± 1 °C.

After one week, the isolation frequency (%) of colonies with a morphology compatible with *Colletotrichum* was determined using the following formula: $F = (N_{Coll}/N_{Tot}) \times 100$, where F is the frequency of *Colletotrichum*, N_{Coll} is the number of woody fragments from which *Colletotrichum*-like colonies were isolated and N_{Tot} is the total number of woody fragments from which fungi were isolated.

Mycelial plugs taken from the actively growing margins of colonies were transferred onto fresh PDA to obtain pure cultures. Single-spore isolates were obtained from the colonies and transferred to new PDA plates. The fungal isolates were stored in sterile distilled water at the Di3A laboratory for further analyses.

2.2. DNA Extraction, PCR Amplification and Sequencing

The total DNA was extracted from all isolates using the E.Z.N.A fungal DNA Mini-Kit (Omega Bio-Tek, Darmstadt, Germany) according to the manufacturer's guidelines. To determine the species complex (SC) of each isolate, an initial PCR amplification was performed targeting the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene on all the obtained isolates. This analysis was used exclusively for an exploratory and provisional assignment of isolates to species complexes and not for definitive species-level identification. For more detailed taxonomic analyses and identification at the species level, 11 representative isolates (APIN 3, APIN 4, APIN 7, APIN 8, APIN 9, APIN 10, APIN 13, APIN 14, APIN 16, APIN 20 and APIN 21) from the two groups identified within the *C. gloeosporioides* SC were further analyzed by sequencing additional genomic regions, including *chs-1*, *act*, *tub2*, *cal*, *gs* and ApMat loci. Representative isolates were selected based on colony morphology and their preliminary genetic grouping inferred from the *gapdh*-based clustering, with the aim of capturing the phenotypic and genetic diversity among the analyzed isolates. The partial *gapdh* was amplified with the primers GDF1–GDR1 [34], while the amplification of *chs-1* and *act* was performed using the primers CHS-79F–CHS-354R and ACT-512F–ACT-783R, respectively [35]. Primers T1 [36]–Bt2-b [37] were used to amplify the partial *tub2* gene, and primers CL1C–CL2C were used for the amplification of the *cal* region [4]. The gs and ApMat genes were amplified using GSF1–GSR1 and AMF1–AMR1, respectively [38]. The PCR amplification mixtures and cycling conditions adopted for all the loci were selected according to previously published protocols [38,39], using Qiagen Taq DNA polymerase (Hilden, Germany), with the final reagent concentrations and cycling parameters detailed in Table S1. PCR amplification was evaluated by running 5 µL of PCR product per reaction on a 1% agarose gel (VWR Life Science AMRESCO® biochemicals, Solon, OH, USA) stained with GelRed™ (Biotium, Fremont, CA, USA) and separated by electrophoresis. Sequencing of the PCR fragments was performed by Macrogen Europe (Milan, Italy), and consensus sequences were obtained through Geneious Prime version 11.0.20.1+1 (Auckland, New Zealand). All the obtained sequences were deposited in Genbank.

2.3. Phylogenetic Analyses

The sequences obtained in this study were initially analyzed using the National Center for Biotechnology Information (NCBI) GenBank database through the standard nucleotide BLAST (blastn) suite (https://blast.ncbi.nlm.nih.gov/, accessed on 10 December 2024). To infer comparative phylogenetic relationships, a total of 87 *Colletotrichum* strains from the *C. gloeosporioides* SC were included as reference taxa, comprising ex-type, epitype and well-characterized reference isolates available in GenBank (Table S2). Each locus was aligned using the MAFFT version 7 online tool (https://mafft.cbrc.jp/alignment/server/index.html, accessed on 23 January 2025) [40] and subsequently refined manually in MEGA version 7 [41]. Alignments were trimmed manually to remove ambiguous regions. No significant topological conflict among loci was detected, validating the concatenation. Phylogenetic reconstructions were carried out using both Maximum Parsimony (MP) and Bayesian Inference (BI) criteria. The MP analyses were conducted with PAUP v.4.0b10 [42], employing heuristic searches with 100 random sequence additions and tree bisection–reconnection branch swapping under equal character weighting. Gaps in the alignments were treated as a fifth character state following a common practice in phylogenetic studies to maximize the phylogenetic signal in insertion–deletion events. The phylogenetic metrics calculated included tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC), with bootstrap support assessed through 1000 replicates [43]. For BI, the best-fit evolutionary model for each locus was identified using MrModeltest v. 2.3 [44] and incorporated into MrBayes v. 3.2.5 [45]. The Markov Chain Monte Carlo algorithm was run with four heated chains (heating parameter: 0.3) and sampling every 1000 generations, starting from a random tree. Runs continued until the average standard deviation of split frequencies dropped below 0.01, ensuring convergence. Sequences generated in the present study were deposited in GenBank (Table S2).

2.4. Pathogenicity Tests

Pathogenicity tests were conducted to evaluate the ability of both species to cause symptoms in avocado plants. As reported by several authors, stem and branch lesions caused by these pathogens may represent a potential source of inoculum for fruit infection [8]. Therefore, additional assays were carried out to assess the pathogenicity of the fungal species in avocado fruits.

The first test was performed in a greenhouse using two-year-old healthy avocado plants (cv. Hass) grafted onto 'Day' rootstocks. One representative isolate of *Colletotrichum persae* (APIN 16) and one of *C. gloeosporioides s.s.* (APIN 3) were used. Six plants were used for each fungal isolate. After disinfecting the stem with 70% ethanol, a wound was made on each plant using a sterile cork borer and a small piece of bark was removed. A mycelial plug (0.5 cm^2) taken from a one-week-old colony of the fungal isolate was placed into the wound, which was then sealed with Parafilm. Control plants were inoculated with PDA plugs. Plants were maintained in the greenhouse for 60 days at temperatures ranging from 18 to 25 °C and approximately 65% relative humidity. Two months after inoculation, mean internal lesion length was measured. To fulfil Koch's postulates, each inoculated isolate was reisolated from symptomatic tissues and *gapdh* sequences were used to confirm the identity of the inoculated fungi. The experiment was conducted twice.

A second test was carried out with one representative isolate of *C. perseae* (APIN 16) and one of *C. gloeosporioides s.s.* (APIN 3) on ten ripe avocado fruits cv. Hass. The fruits were surface-sterilized in 1.5% sodium hypochlorite (NaOCl) for 2 min, rinsed twice in sterile distilled water (SDW) for 1 min and dried on sterile paper. Each fruit was wounded at two points with a sterile micro-needle. Each wound was inoculated with 10 μL of a conidial suspension (1×10^6 conidia/mL). The control consisted of wounded and inoculated fruits with sterile water. Additionally, 10 unwounded fruits for both species were inoculated at the stem end following the procedure described above. Successively, fruits were incubated for five days in a humid growth chamber (about 85% relative humidity) at 25 °C. After 5 days, the mean lesion length based on two perpendicular diameters was measured. To satisfy Koch's postulates, each species was reisolated from symptomatic tissues and identified as described for the tests on plants. The test was repeated once.

3. Results

3.1. Field Sampling and Fungal Isolation

During the period between April and May, new symptoms were observed on young plants cv. Hass after grafting on 'Day' in the nursery with a relative humidity from 75 to 85% and temperatures from 15 to 24 °C. Disease incidence was observed in more than 45% (of about 20,000 plants). All symptomatic plants showed necrotic lesions on shoots and stems, as well as dieback, often starting at the grafting point and causing the death of the plant (Figure 1). *Colletotrichum*-like fungi were detected in all the samples analyzed with an isolation frequency of 67.5%. A total of 46 *Colletotrichum* isolates were collected and stored as monosporic cultures.

3.2. Phylogenetic Analyses

The combined species phylogeny of the *Colletotrichum* isolates consisted of 98 sequences, including the outgroup sequences of *C. pyrifoliae* (CGMCC 3.18902). A total of 3832 characters (*gapdh*: 1–267, *chs-1*: 274–502, *act*: 509–750, *tub2*: 757–1,267, *cal*: 1274–1972, *gs*: 1979–2917 and ApMat: 2924–3832) were included in the phylogenetic analysis. A total of 951 characters were parsimony-informative, 1205 were variable and parsimony-uninformative and 1640 were constant. A maximum number of 1000 equally most parsimonious trees were saved (tree length = 3827, CI = 0.741, RI = 0.875 and RC = 0.649). Bootstrap support values obtained with the parsimony analysis are shown in the Bayesian phylogeny in Figure 2. For the Bayesian analysis, the Dirichlet state frequency distributions were suggested by MrModeltest to analyze the *gapdh*, *act*, *tub2* and *gs* partitions, whilst base frequencies were fixed as equal for the *chs-1*, *cal* and ApMat partitions. The following models, recommended by MrModeltest, were used: GTR+G for *tub2*, HKY+G for *gapdh*, *act* and *gs*, K80+G for *chs-1* and ApMat, and SYM+G for *cal*. In the BI analysis, the partitions exhibited the following numbers of unique site patterns: *gapdh* 206, *chs-1* 86, *act* 163, *tub2* 268, *cal* 205, *gs* 437 and ApMat 523. The run was for 18,590,000 generations, resulting in 37,182 trees, of which 27,888 trees were sampled to calculate the posterior probabilities. In the combined analyses, six isolates (APIN 8, APIN 9, APIN 10, APIN 16, APIN 20 and APIN 21) grouped with four reference strains, including the epitype, of *C. perseae* in a strongly supported cluster, whilst five isolates (APIN 3, APIN 4, APIN 7, APIN 13 and APIN 14) formed a distinctly supported cluster with reference strains of *C. gloeosporioides s.s.*, including the epitype and three species recently synonymized under this taxon.

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Figure 1. Symptoms caused by *Colletotrichum* species in young avocado plants in nursery: (**a**) infected discarded plants; (**b**) necrotic lesion on stem; (**c**,**d**) lesions originating from grafting points.

Figure 2. Consensus phylogram resulting from a Bayesian analysis of the combined *gapdh*, *chs*, *act*, *tub2*, *cal*, *gs* and ApMat sequences of *Colletotrichum* spp. Bayesian posterior probability values (BI analysis) and bootstrap support values (MP analysis) are indicated at the nodes. The isolates collected in this study are in bold. The tree was rooted to *C. pyrifoliae* (CGMCC 3.18902).

3.3. Pathogenicity Tests

After two months, external necrotic lesions, wood discoloration and reddish-brown streaking on the stem at the inoculation point were induced in both species, whereas no symptoms developed on the control plants (Figure 3). External necrotic lesions measured, on average, 3.2 cm for *C. perseae* (APIN16) and 2.5 cm for *C. gloeosporioides s.s.* (APIN3). In addition, wood discoloration and reddish-brown streaking were observed both above and below the necrotic area, extending along the stem, with mean lesion lengths of 12.0 cm for isolate APIN16 and 12.6 cm for APIN3.

In fruit under controlled experimental conditions, after peeling, circular brown rot was observed in the pulp, extending deep into the fruit tissue (Figure 3). After five days, the average lesion diameters were 2.5 cm for APIN16 and 2.4 cm for APIN3, with lesions extending to a depth of approximately 1 cm. In addition, both fungi produced acervuli (asexual fruiting bodies) on inoculated fruits.

Fruit inoculated at the stem end with both fungal isolates developed a circular brown rot at the inoculation site, with mycelium and acervuli forming in the central region.

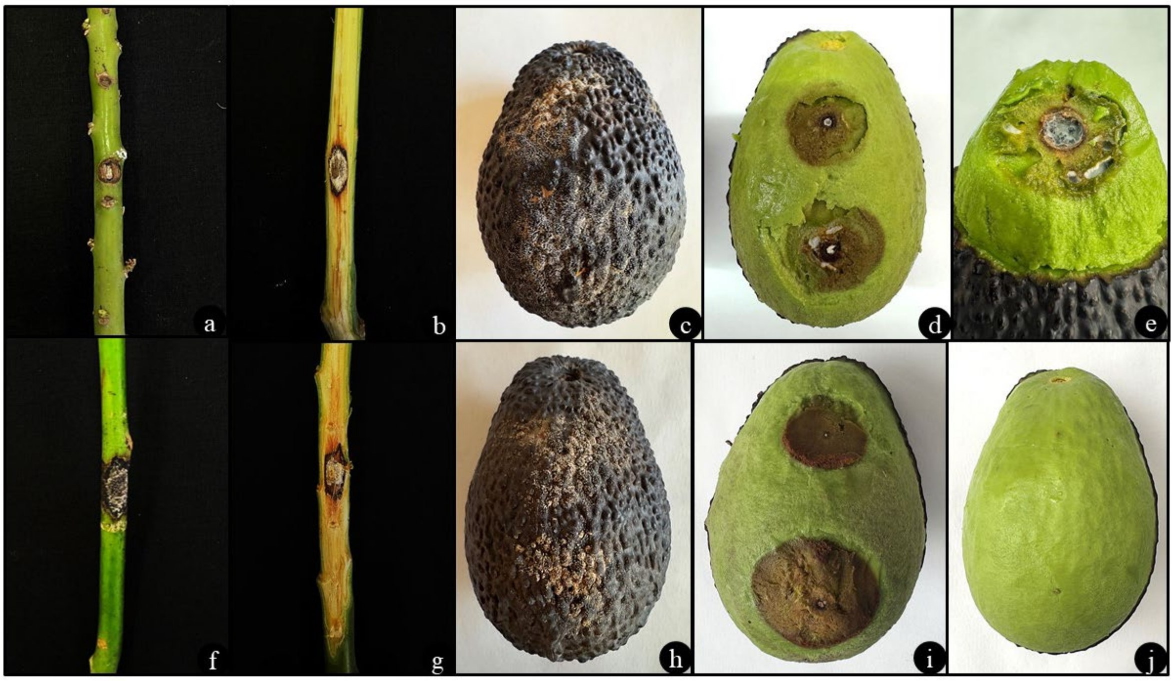

Figure 3. Pathogenicity tests performed on avocado stems and fruit: (**a**,**b**) external and internal necrotic lesion on stem caused by *Colletotrichum gloeosporioides s.s.*; (**c**,**d**) conidia and acervuli of *C. gloeosporioides s.s.* and internal fruit rot caused by infection; (**e**) rot on stem-end of fruit caused by *C. gloeosporioides s.s.* and visible mycelium and acervuli; (**f**,**g**) external and internal necrotic lesion on stem caused by *C. perseae*; (**h**,**i**) conidia and acervuli of *C. perseae* and internal fruit rot caused by infection; (**j**) control fruit.

4. Discussion

The present study expands the current knowledge of fungal diseases affecting avocado plants in Europe, with a focus on Italy. Here, species belonging to the genus *Colletotrichum*, i.e., *Colletotrichum gloeosporioides* (*s.s.*) and *C. perseae*, were identified as causal agents of necrotic lesion and dieback in young avocado plants cv. Hass following grafting in a nursery in Sicily.

Previous studies have highlighted the presence of several fungal diseases affecting avocado branches, stems and shoots and shown an increased incidence of infections in avocado orchards [12,29,30,32,46,47]. However, no *Colletotrichum* species causing aerial symptoms has previously been reported.

Colletotrichum species are known for their ability to cause anthracnose on avocado worldwide, leading to significant pre- and postharvest losses [7,8,25]. Recent molecular studies have revealed that the diversity of *Colletotrichum* species associated with avocado is greater than previously assumed, extending beyond the species *C. gloeosporioides* and *C. acutatum* [15–17].

In Italy, *C. gloeosporioides s.s.* has previously been reported as a causal agent of stem-end rot of avocado fruit, together with *C. fructicola*, another member of the *C. gloeosporioides* SC. In addition, *C. fioriniae*, belonging to the *C. acutatum* SC, has been associated with postharvest fruit rot [11]. *Colletotrichum perseae* has been reported in New Zealand and Israel in association with anthracnose symptoms in avocado fruit, together with other *Colletotrichum* spp. [9,10,14]. This study is the first report of *C. perseae* in Europe and provides the first evidence of its role in causing necrotic lesions and shoot dieback in avocado plants.

The identification of *Colletotrichum* spp. in young avocado plants at the nursery stage further supports the hypothesis, previously proposed by Mc Donald [25], that infections can emerge at an early stage, including during grafting, ultimately leading to the death of plants. The detection of *Colletotrichum* inoculum in an Italian avocado nursery confirms the possibility of these pathogens being transmitted through infected propagation material [25]. In this regard, a recent study demonstrated that the highest inoculum levels of *C. gloeosporioides s.l.* are present in avocado embryos and cotyledons, while lower levels occur in the germination substrate [48].

In our study, the high disease severity and substantial plant mortality observed in the nursery highlight the importance of early monitoring and greater attention to prevent inoculum dissemination during propagation process, especially during grafting. This phase is crucial for healthy plant production and requires the use of healthy propagation material collected from inspected healthy plants. In addition, other factors contributing to disease development include the high inoculum pressure in the nursery, favorable environmental conditions (warm and humid weather) and agronomic practices that create wounds, facilitating pathogen entry. As reported by different authors, *Colletotrichum* inoculum (acervuli and perithecia) can be present on both living and dead tissues of branches, stems and leaves [25], making inoculum management particularly challenging for growers.

According to several authors, both the frequency of *Colletotrichum* isolation and disease severity can vary among species [14,17]. The results of our studies confirmed that both tested *Colletotrichum* species are able to cause wood lesions and fruit rot with similar results. Unlike previous reports describing *Colletotrichum* spp. as secondary invaders of avocado wood tissues [20,21], our findings highlight their primary role in nursery infections and their pathogenic potential for causing fruit infections during the postharvest stage.

Overall, given the role of *Colletotrichum* species as primary pathogens in Italian nursery infections in avocado plants cv. Hass and their ability to cause fruit rot, accurate species-level identification is essential. *Colletotrichum* is a cryptic genus that includes several morphologically similar species that cannot be reliably distinguished based on morphology alone. Therefore, multi-locus phylogenetic analyses, including several informative loci, are required for precise species identification [39,49]. Precise species delineation is crucial for assessing pathogen diversity, understanding differences in epidemiology and interactions with environmental factors, and gaining insight into infection mechanisms, ultimately supporting the development of targeted management strategies in both nursery and postharvest contexts.

This research contributes to a deeper understanding of *Colletotrichum* diversity and pathogenicity in avocado in Italy. The emergence of *Colletotrichum* species in nursery propagation materials further expands the spectrum of the diseases affecting this crop in Italy and indicates a dynamic and evolving etiological picture. Strict propagation protocols and early detection through molecular diagnostic methods are crucial to minimizing infection risks and to ensure the establishment of healthy avocado orchards.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae12010111/s1: Table S1: List of amplified loci, corresponding primer names and sequences, and full PCR conditions applied in this study; Table S2: Collection details and GenBank accession numbers of isolates included in this study.

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Data Availability Statement: The original contributions presented in this study are included in the article. Further requests can be directed to the corresponding author.

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