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Colletotrichum fioriniae, causal agent of postharvest avocado fruit rot in Southern Italy

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Summary. *Colletotrichum* includes pathogens affecting different plant hosts in tropical, subtropical, and temperate regions. Anthracnose caused by these pathogens is a prevalent and severe postharvest disease of tropical fruits, including avocado. In 2021, avocado fruit with typical anthracnose symptoms was found during storage, and on very ripe fruit, in Caserta, Campania region, Italy. Avocado cultivation and production is increasing in this region, so the etiology of this disease was examined. Molecular and phylogenetic analyses on four genomic loci (ITS, *gapdh*, *act* and *tub2*) combined with morphology identified selected representative isolates as *Colletotrichum fioriniae*, in the *C. acutatum* species complex. Pathogenicity of isolates was confirmed by inoculating them on healthy avocado fruit (cv. Pinkerton). This is the first report of *C. fioriniae* causing post-harvest fruit rot on avocado in Italy. This pathogen merits further epidemiological and ecological investigations, to provide basic knowledge supporting development of management of its spread and mitigating possible impacts on avocado production.

Keywords. Anthracnose, *Colletotrichum acutatum* species complex, *Persea americana.*

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INTRODUCTION

Avocado (*Persea americana* Mill.) is native to Mexico, and is cultivated in tropical and subtropical regions, with Spain, Portugal and Greece as the three major European countries for commercial production (FAOSTAT, 2024). In Italy, avocado plants were initially introduced for ornamental purposes; then, they began to be cultivated along the coasts, where climatic conditions were favourable for their growth (Guarnaccia *et al.*, 2016). During recent years, the increasing demand of avocado fruit, mainly due to their health benefits, has resulted in increased avocado cultivation, with Sic-

ily, Calabria, Campania, Puglia and Sardinia currently being main productive regions of the Italian peninsula (Migliore *et al*., 2017).

Colletotrichum pathogens are ranked eighth in terms of economic damage and importance, causing anthracnose, leaf spots, shoot dieback and postharvest rot and decay on many tropical and temperate fruit types (Dean *et al*., 2012; Aiello *et al.,* 2015; Guarnaccia *et al*., 2021a; 2021b). Twenty *Colletotrichum* spp., belonging to the gloeosporioides, acutatum, boninense, gigasporum, dematium and magnum Species Complexes (SC), have been reported affecting avocado in the main productive countries, including Australia, Chile, China, Colombia, Israel, Italy, Mexico, Turkey and New Zealand (Guarnaccia *et al*., 2016; Sharma *et al*., 2017; Giblin *et al*., 2018; Fuentes-Aragón *et al*., 2020; Hofer *et al*., 2021; Bustamante *et al*., 2022; USDA, Fungal Database, 2024). Symptoms on avocado leaves are usually yellow spots and necrotic lesions at the tips or between veins. Fruit symptoms develop around lenticels as small brown to black lesions that become larger and sunken after harvest (Talhinhas and Baroncelli, 2023).

In March 2021, avocado fruit originating from trees cultivated in an experimental orchard at the Centre for Olive, Fruit and Citrus Crops located in Caserta (Campania, Italy) showed typical symptoms of anthracnose and *Colletotrichum* spp. infections after 10 d storage at 18 ± 2°C. Because of the diversity of *Colletotrichum* spp. reported on avocado, the present study aimed to identify and characterize the causal agent of this post-harvest disease, through molecular and phylogenetic analyses, assessment of morphological features, and pathogenicity tests.

MATERIALS AND METHODS

Isolation and morphological observations

Forty symptomatic mature avocado fruits were obtained from the experimental orchard at the Centre for Olive, Fruit and Citrus Crops, located in Caserta (41°04'24.5"N, 14°19'04.7"E). Small sections (3×3 mm diam.) were excised with a scalpel from the margins of circular spots. The fragments were surface disinfected three times in a 2% sodium hypochlorite solution for 1 min, then rinsed in sterile distilled water (SDW) for 1 min, dried in laminar flow cabinet, and transferred onto potato dextrose agar (PDA, Oxoid) amended with 100 mg L^{-1} streptomycin sulphate (Merck) in Petri plates. Conidia were also harvested from the conidial masses developing on 80% of the field-collected symptomatic fruits and placed on PDA-S. All the plates were incubated in the dark at 24 ± 2°C for 2 to 3 d. Developing colonies morphologically resembling *Colletotrichum* spp. were placed on new PDA-S plates. After 7 d, single conidium cultures were established. A total of ten isolates was obtained, and two representative isolates were used for analyses.

Morphological features of the isolates were observed on the growing colonies using a light microscope (Olympus BH-2). Culture colours were determined according to Rayner (1970). Conidia were examined by mounting fungal structures in sterile water and the length and width of 30 conidia were measured for each isolate using a light microscope at ×40 magnification. The average dimensions and standard deviations were calculated.

Molecular characterization and phylogenetic analyses

DNA was extracted from two representative isolates (GP1 and GP2) using the DNeasy Plant Mini Kit (Qiagen). The PCR reactions were each carried out with 10 ng of DNA template, with the primer pair ITS1/ITS4 (White *et al.,* 1990) to amplify the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA operon, including the 3' end of the 18S rRNA, the first internal transcribed spacer region, the 5.8S rRNA gene, the second internal transcribed spacer region and the 5' end of the 28S rRNA gene. The primer pair Btub2Fd/Btub4Rd was used for amplification of part of the β-tubulin II region (Stielow *et al.,* 2015). The primer pair GDF1/GDR1 (Guerber *et al.,* 2003) was used to amplify the glyceraldehyde-3-phosphate dehydrogenase locus, and ACT512F/ACT783R (Carbone and Kohn, 1999) was used to amplify actin locus. The reactions were each prepared in a total volume of 50 µL containing 1× of reaction buffer, 200 µM of dNTPs set, 500 mM of each primer, and 1 unit of Q5 Hi-Fi DNA polymerase (New England Biolabs). Reaction tubes were incubated in the thermocycler, at 94°C for 1 min, followed by 35 cycles of 20 s at 94°C, 30 s at 58–62°C and 30 s at 72°C, and a final extension at 72°C for 5 min. Amplicons were purified using the Nucleospin Extract- Kit (Macherey-Nagel), and were sequenced by BMR Genomics (Padova, Italy). The nucleotide sequences were analysed by BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the default parameters described by Altschul *et al.* (1990). Reference sequences were retrieved among the closest relative species, and *Colletotrichum* spp. reported as pathogens of *P. americana* were also retrieved to obtain a taxonomic framework of the analysed isolates.

The sequences obtained were initially aligned with reference sequences using MAFFT v. 7 online server (https://mafft.cbrc.jp/alignment/server/index.html)

(Katoh and Standley, 2013) for each considered locus. A manual adjustment in MEGA v. 7 (Kumar *et al.*, 2016) was then made to determine multi-locus phylogenies based on Maximum Parsimony (MP) and Bayesian Inference (BI) criteria. Phylogenetic Analysis Using Parsimony (PAUP) v.4.0b10 (Swofford, 2003) was used for MP phylogeny. Heuristic searches with 100 random additional sequences estimated the phylogenetic relationships. Tree bisection-reconnection was used. The branch swapping option was set on "best trees" and all characters were weighted equally. Alignment gaps were considered as "fifth base". The analysis calculated the following parameters: tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC). A total of 1000 replications were set for the bootstrap analyses (Hillis and Bull, 1993). For the BI analysis, MrModeltest v.2.3 (Nylander, 2004) was run to determine the best evolutionary model for each considered locus. The determined model was incorporated in the analyses performed with MrBayes v. 3.2.5 (Ronquist *et al.*, 2012) to generate phylogenetic trees, following optimal criteria for each locus. The Markov Chain Monte Carlo (MCMC) analysis started from a random tree topology, and four chains were used. The heating parameter was set at 0.3 and the sampling frequency at 1000 generations. Analyses stopped when the average standard deviation of split frequencies was below 0.01. Sequences generated in the present study were deposited in GenBank (Table 1).

Pathogenicity tests

Pathogenicity tests were carried out using the isolates GP1 and GP2, to assess fulfilment of Koch's postulates. The isolates were inoculated on wounded (1 mm diam.) fruit of *P. americana* cv. Pinkerton. The trial was conducted using ten fruit for each tested isolate. A total of 20 fruit for each isolate were washed, dipped in 1.5% sodium hypochlorite for 30 sec, rinsed twice in sterile distilled water (SDW) for 1 min and then dried on absorbent paper. Each fruit was wounded at three points with a sterile needle, and for each wound 5 µL of conidium suspension (10^4 conidia mL^{-1}) was applied. Conidium suspensions of each isolate were prepared by adding SDW to 10 day old cultures grown on PDA-S, scraping the mycelia and conidium masses and filtering the mixture through muslin cloth. Inoculation control fruits were each treated with 5 µL of SDW. After inoculation, the fruits were placed in plastic containers covered with plastic film, and were incubated in growth chamber at 25°C 100% RH for 3 d. The assay was carried out two times.

RESULTS AND DISCUSSION

The collected fruit showed depressed circular spots, and developed orange-pink mucilaginous lumps after storage (Figure 1). The fungal colonies obtained from isolations and directly from conidium masses were identified as *Colletotrichum* spp., based on their morphologies. The cultures of the two representative isolates (GP1 and GP2) had light grey to pink mycelium with abundant salmon pink masses of conidia on the upper surfaces, and pinkish mycelium with black spots on the reverse surfaces. The conidia were hyaline, narrowly elliptical, with guttulae, and measured (mean ± SD length and width) 13 ± 4.5 × 3.3 ± 1.6 µm.

The multi-locus phylogeny, which allowed precise identification of the two representative isolates, consisted of 57 sequences, including the outgroup (*Monilochaetes infuscans*, CBS 896.96), and included a total of 1709 characters (ITS: 1–581; *gapdh*: 588–895; *act*: 902–1183; *tub2*: 1190–1709). A total of 565 characters was parsimony-informative, 349 characters were variable and parsimony-uninformative, and 767 were constant. A maximum number of 1000 equally most parsimonious trees was saved (Tree length = 2255, CI = 0.659, RI = 0.900 and RC = 0.593). Bootstrap values obtained with the MP analysis are indicated on the BI phylogenetic tree in Figure 2. For the BI analysis, the following models, recommended by MrModeltest, were used: GTR + I + G for ITS, HKY + G for *gapdh*, and GTR + G for *act* and *tub2*. In the BI analysis, the ITS locus had 159 unique site patterns, the *gapdh* locus had 255, the *act* locus had 162, and the *tub2* locus had 267 unique site patterns. The analysis ran for 1,550,000 generations, resulting in 3102 trees, of which 2327 were used to calculate the posterior probabilities. In the multi-locus analyses, the isolates GP1 and GP2 obtained from the present study clustered with three reference strains of *C. fioriniae*.

For the pathogenicity assessments, all the inoculated fruits showed symptoms similar to those observed on the sample collected in the orchard (Figure 1), while non-inoculated control fruit did not show symptoms. After 10 d post inoculation, all the fruits inoculated with the two isolates were completely rotten. The inoculated fungi were successfully re-isolated from lesions of the inoculated fruits, fulfilling Koch's postulates.

The genus *Colletotrichum* includes 340 recognised species, which are grouped into 20 species complexes, and several species belonging to this genus are pathogens of cultivated plants in tropical, subtropical and temperate regions (Dean *et al.,* 2012). In particular, anthracnose caused by *Colletotrichum* spp. is the most widespread and serious postharvest disease of many

Table 1. Details of *Colletotrichum* isolates used in phylogenetic analyses, and their corresponding GenBank accession numbers. Isolates from the present study are indicated in bold font.

Species	Code*	Country	Host species	Genbank Accession Number			
				ITS	*tub2*	*act*	*gapdh*
Colletotrichum acutatum	CBS 112996 t	Australia	*Carica papaya*	JQ005776	JQ005860	JQ005839	JQ948677
Colletotrichum aenigma	ICMP 18608 t	Israel	*Persea americana*	JX010244	JX010389	JX009443	JX010044
Colletotrichum aeschynomenes	ICMP 17673 t	USA	*Aeschynomene virginica*	JX010176	-	JX009483	JX009930
Colletotrichum alatae	ICMP 17919	India	*Dioscorea alata*	JX010190	-	JX009471	JX009990
Colletotrichum alienum	ICMP 12071 t	New Zealand	*Malus domestica*	JX010251	-	JX009572	JX010028
Colletotrichum asianum	ICMP 18580, CBS 130418	Thailand	*Coffea arabica*	JX010196	-	JX009584	JX010053
Colletotrichum brisbanense	CBS 292.67 t	Australia	*Capsicum annuum*	JQ948291	JQ949942	JQ949612	JQ948621
Colletotrichum chrysanthemi	CBS 126518	The Netherlands	*Carthamus* sp.	JQ948271	JQ949922	JQ949592	JQ948601
Colletotrichum chrysophilum	CMM4268 t	Brazil	*Musa* sp.	KX094252	KX094285	KX093982	KX094183
Colletotrichum cigarro	ICMP 18539 t	Australia	*Olea europaea*	JX010230	JX010434	JX009523	JX009966
Colletotrichum clidemiae	ICMP 18658	USA (Hawai)	*Clidemia hirta*	JX010265	-	JX009537	JX009989
Colletotrichum cordylinicola	ICMP 18579 t	Thailand	*Cordyline fructicosa*	JX010226	JX010440	JX009586	JX009975
Colletotrichum cosmi	CBS 853.73 t	The Netherlands	*Cosmos* sp.	JQ948274	JQ949925	JQ949595	JQ948604
Colletotrichum costaricense	CBS 330.75 t	Costa Rica	*Coffea arabica*	JQ948180	JQ949831	JQ949501	JQ948510
Colletotrichum cuscutae	IMI 304802 t	Dominica	*Cuscuta* sp.	JQ948195	JQ949846	JQ949516	JQ948525
Colletotrichum fioriniae	ATCC 28992	USA	*Malus domestica*	JQ948297	JQ949948	JQ949618	JQ948627
Colletotrichum fioriniae	CBS 129916	USA	*Vaccinium* sp.	JQ948317	JQ949968	JQ949638	JQ948647
Colletotrichum fioriniae	CBS 293.67	Australia	*Persea americana*	JQ948310	JQ949961	JQ949631	JQ948640
Colletotrichum fioriniae	**GP1**	**Italy**	***Persea americana***	**MZ613321**	**PQ295819**	**OR767286**	**OR767287**
Colletotrichum fioriniae	**GP2**	**Italy**	***Persea americana***	**PQ283879**	**PQ295820**	**PQ295821**	**PQ295822**
Colletotrichum gloeosporioides	ICMP 17821, CBS 112999	Italy	*Citrus sinensis*	JX010152	JX010445	JX009531	JX010056
Colletotrichum godetiae	CBS 133.44 t	Denmark	*Clarkia hybrida*	MH856119	JQ950053	JQ949723	JQ948733
Colletotrichum guajavae	IMI 350839 t	India	*Psidium guajava*	JQ948270	JQ949921	JQ949591	JQ948600
Colletotrichum horii	C1180.1	Japan	*Diospyros kaki*	GQ329690	JX010450	JX009533	GQ329685
Colletotrichum indonesiense	CBS 127551 t	Indonesia	*Eucalyptus* sp.	JQ948288	JQ949939	JQ949609	JQ948618
Colletotrichum jiangxiense	GCMCC 3.17363 t	China	*Camellia sinensis*	NR_152279	OK236389	-	-
Colletotrichum karsti	CBS 106.91	Brazil	*Carica papaya*	JQ005220	JQ005654	JQ005568	JQ005307
Colletotrichum kinghornii	CBS 198.35 t	UK	*Phormium* sp.	JQ948454	JQ950105	JQ949775	JQ948785
Colletotrichum laticiphilum	CBS 112989 t	India	*Hevea brasiliensis*	JQ948289	JQ949940	JQ949610	JQ948619
Colletotrichum limetticola	CBS 114.14 t	USA	*Citrus aurantifolia*	JQ948193	JQ949844	JQ949514	JQ948523
Colletotrichum lupini	CBS 109225 t	Ukraine	*Lupinus albus*	JQ948155	JQ949806	JQ949476	JQ948485
Colletotrichum melonis	CBS 159.84 t	Brazil	*Cucumis melo*	JQ948194	JQ949845	JQ949515	JQ948524
Colletotrichum nupharicola	CBS 470.96	USA	*Nuphar polysepala*	JX010187	JX010398	-	-
Colletotrichum nymphaeae	CBS 100065	The Netherlands	*Anemone* sp.	JQ948225	JQ949876	JQ949546	JQ948555
Colletotrichum nymphaeae	CBS 129928	USA	*Fragaria* × *ananassa*	JQ948228	JQ949879	JQ949549	JQ948558

(Continued)

Table 1. (Continued).

Species	Code*	Country	Host species	Genbank Accession Number			
				ITS	*tub2*	*act*	*gapdh*
Colletotrichum nymphaeae	CBS 231.49	Portugal	*Olea europaea*	JN121201	JN121288	JQ949523	JQ948532
Colletotrichum nymphaeae	IMI 370491	Brazil	*Malus pumila*	JQ948204	JQ949855	JQ949525	JQ948534
Colletotrichum orchidophilum	CBS 632.80 t	USA	*Dendrobium* sp.	JQ948151	JQ949802	JQ949472	JQ948481
Colletotrichum paxtonii	IMI 165753 t	Saint Lucia	*Musa* sp.	JQ948285	JQ949936	JQ949607	JQ948615
Colletotrichum perseae	CBS 141365	Israel	*Persea americana*	KX620308	KX620341	KX620145	KX620242
Colletotrichum phormii	CBS 118194 t	Germany	*Phormium* sp.	JQ948446	JQ950097	JQ949768	JQ948777
Colletotrichum pseudoacutatum	CBS 436.77 t	Chile	*Pinus radiata*	JQ948480	JQ950131	JQ949801	JQ948811
Colletotrichum psidii	CBS 145.29 t	Italy	*Psidium* sp.	JX010219	JX010443	JX009515	JX009967
Colletotrichum pyricola	CBS 128531 t	New Zealand	*Pyrus communis*	JQ948445	JQ950096	JQ949766	JQ948776
Colletotrichum queenslandicum	ICMP 1778	Australia	*Carica papaya*	JX010276	JX010414	JX009447	JX009934
Colletotrichum rhombiforme	CBS 129953 t	Portugal	*Olea europaea*	JQ948457	JQ950108	JQ949778	JQ948788
Colletotrichum roseum	INIA<CHL>:RGM 2685	Chile	*Lapageria rosea*	MK903611	MK903607	MK903604	MK903603
Colletotrichum salicis	CBS 607.94 t	The Netherlands	*Salix* sp.	JQ948460	JQ950111	JQ949781	JQ948791
Colletotrichum salsolae	ICMP 19051	Hungary	*Salsola tragus*	JX010242	JX010403	JX009562	JX009916
Colletotrichum scovillei	CBS 126529 t	Indonesia	*Capsicum* sp.	JQ948267	JQ949918	JQ949588	JQ948597
Colletotrichum siamense	ICMP 18578	Thailand	*Coffea arabica*	JX010171	-	JX009518	JX009924
Colletotrichum simmondsii	CBS 122122 t	Australia	*Carica papaya*	JQ948276	JQ949927	JQ949597	JQ948606
Colletotrichum theobromicola	CBS 124945	Panama	*Theobroma cacao*	JX010294	JX010447	JX009444	JX010006
Colletotrichum tropicale	ICMP 18653	Panama	*Theobroma cacao*	JX010264	JX010407	JX009489	JX010007
Colletotrichum walleri	CBS 125472 t	Vietnam	*Coffea* sp.	JQ948275	JQ949926	JQ949596	JQ948605
Colletotrichum xanthorrhoeae	BRIP 45094 t	Australia	*Xanthorrhoea preissii*	JX010261	JX010448	JX009478	JX009927
Monilochaetes infuscans	CBS 869.96	South Africa	*Ipomoea batatas*	JQ005780	JQ005864	JQ005843	JX546612

* t = type culture.

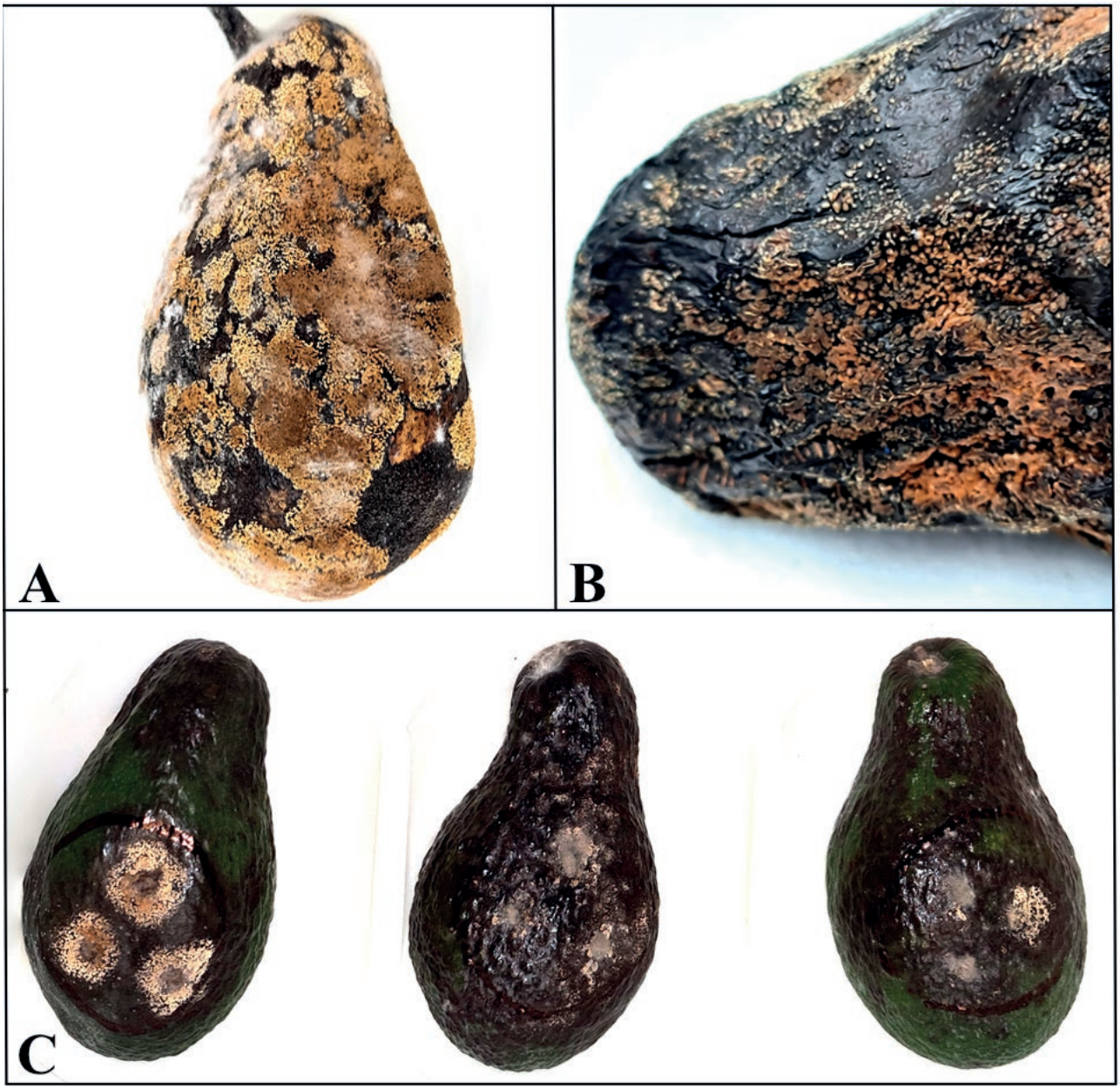

Figure 1. A and **B**, Symptomatic avocado fruit (cv. Pinkerton), showing sunken necrotic lesions and conidium masses on fruit epidermis; **C**, Avocado fruits (cv. Pinkerton) showing symptoms 7 d after inoculation with isolate GP1 of *Colletotrichum fioriniae*.

tropical fruits including avocado, lychee, mango and papaya (Zakaria, 2021; Talhinhas and Baroncelli, 2023).

Investigating the etiology of anthracnose has presented several challenges. The most reliable methods for identifying species within *Colletotrichum* involve the use of multi-locus phylogenies combined with morphological feature assessments (Bhunjun *et al*., 2021). In the present study, the molecular and phylogenetic analyses, and morphological assessments identified the isolates found in association with avocado fruit rot as belonging to *C. fioriniae*. In addition, pathogenicity of this species on avocado fruit was demonstrated. This is the first report of *C. fioriniae* infecting avocado in Italy.

Colletotrichum fioriniae has been previously identified from avocado in Mexico and Australia (Fuentes-Aragón *et al*., 2020; USDA Fungal Database, 2024). In general, in recent years, *C. fioriniae* has been reported as the causal agent of fruit rot and leaf spots on sev-

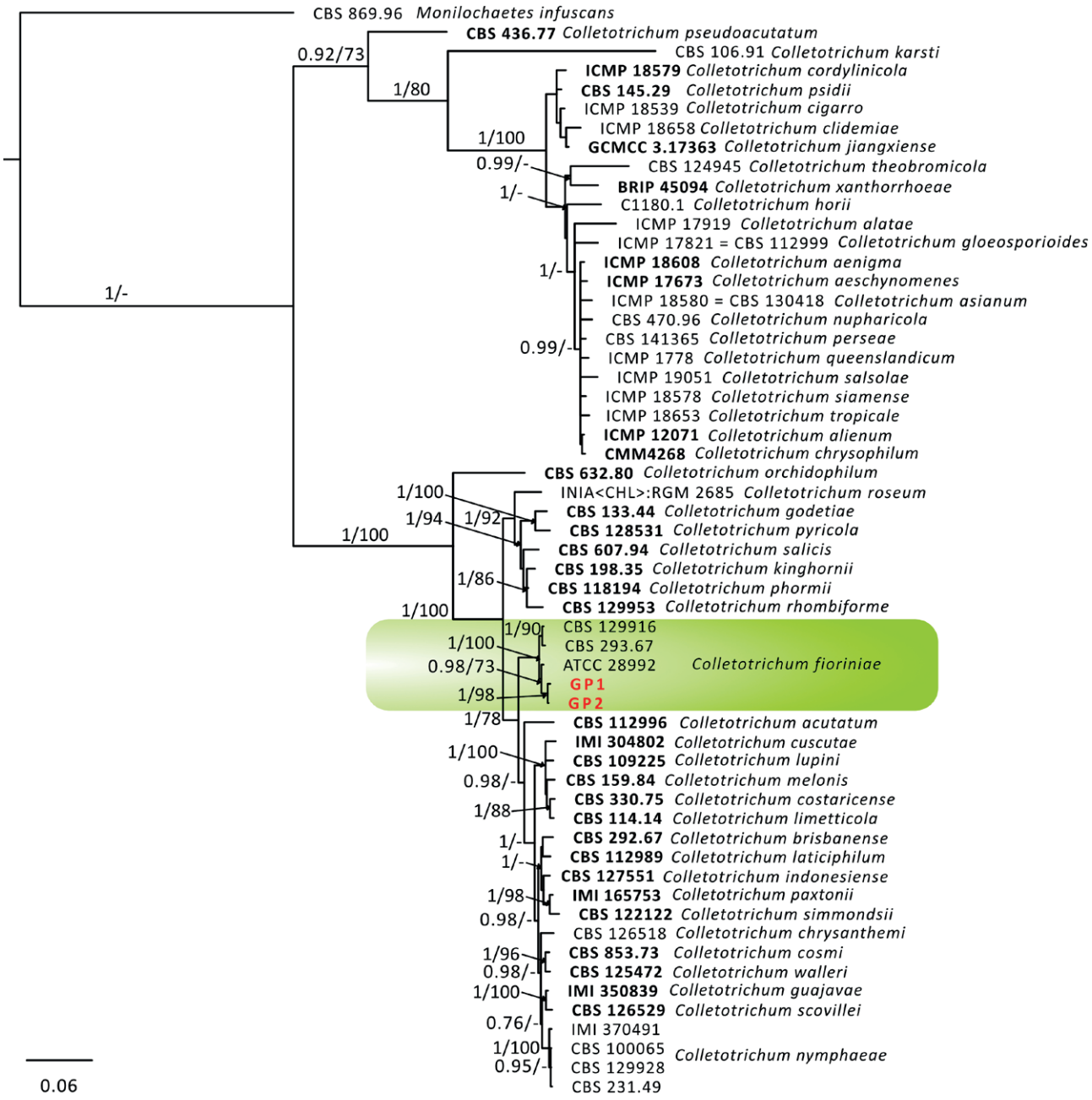

Figure 2. Consensus phylogram resulting from a Bayesian analysis of the combined ITS, *gapdh*, *act* and *tub2* sequences of *Colletotrichum* spp. Bayesian posterior probability values (BI) and Bootstrap support values (MP) are indicated at the nodes. The isolates collected and species found in the present study are indicated in red font. Ex-type strains are indicated in bold. The tree was rooted to *Monilochaetes infuscans* (CBS 869.96).

eral plant hosts, including apple, pear, peach, walnut, hazelnut and tomato (Kou *et al.,* 2014; Munda, 2014; Sezer *et al.,* 2017; Chechi *et al.,* 2019; USDA, Fungal Database, 2024). In Italy, this fungus has been reported as the causal agent of apple bitter rot (ABR), leaf spots on sage (*Salvia leucantha)* and mahonia (*Mahonia aquifolium*), and anthracnose on walnut (Guarnaccia *et al.,* 2019; 2021a; Carneiro and Baric, 2021; Luongo *et al.,* 2022). Several of the above mentioned horticultural crops are important crops cultivated in Campania region. Thus, *C. fioriniae* infecting avocado poses a serious threat to cultivation of this host, and to other relevant host crops with possible significant economic impacts on their supply chains. Latent infec-

tions not detectable at harvest may also occur, increasing difficulty to minimize losses caused by *Colletotrichum* pathogens (De Silva *et al*., 2017). Further investigations should be carried out to determine possible cross-infection patterns considering other possible hosts, and to understand effects of the mild climate of Southern Italy on plant-pathogen interactions and disease development to investigate the epidemiology and eventual spread of *C. fioriniae*.

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