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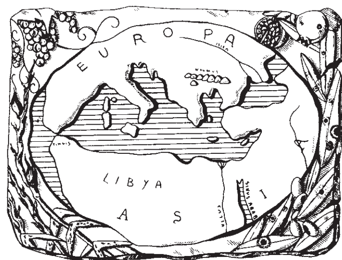

PHYTOPATHOLOGIA MEDITERRANEA

Plant health and food safety

The international journal edited by the Mediterranean Phytopathological Union
founded by A. Ciccarone and G. Goidànich

Phytopathologia Mediterranea is an international journal edited by the Mediterranean Phytopathological Union The journal's mission is the promotion of plant health for Mediterranean crops, climate and regions, safe food production, and the transfer of knowledge on diseases and their sustainable management.
The journal deals with all areas of plant pathology, including epidemiology, disease control, biochemical and physiological aspects, and utilization of molecular technologies. All types of plant pathogens are covered, including fungi, nematodes, protozoa, bacteria, phytoplasmas, viruses, and viroids. Papers on mycotoxins, biological and integrated management of plant diseases, and the use of natural substances in disease and weed control are also strongly encouraged. The journal focuses on pathology of Mediterranean crops grown throughout the world.
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Research Papers

A new disease of kumquat (*Fortunella margarita*) caused by *Colletotrichum karsti*: twig and branch dieback

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Summary. *Citrus* fruit crops are important in many countries. Anthracnose, post bloom fruit drop, fruit stem-end rot, twig and branch dieback and gummosis, caused by *Colletotrichum* spp., are diseases that seriously threaten citrus production. Surveys of kumquat (*Fortunella margarita*) orchards were conducted in Eastern Sicily, Southern Italy, during 2022-23. Fungi isolated from twig and branch dieback of *F. margarita* were identified as *Colletotrichum karsti* through multi-locus (*gapdh*, *tub2* and *act*) phylogeny. Pathogenicity and aggressiveness on detached apple fruit and kumquat plants were confirmed for a selection of representative isolates, although with different levels of disease incidence observed. This is the most comprehensive study on identification of *C. karsti* as the causal agent of twig and branch dieback of kumquat.

Keywords. Fungal disease, phylogenetic analysis, pathogenicity, aggressiveness.

INTRODUCTION

Rutaceae include widely and economically cultivated plant genera including *Citrus*, *Fortunella* and *Poncirus*. Cultivation of *Citrus* and allied genera occurs in more than 140 countries (Liu *et al*., 2012). Italy is one of the ten major citrus-producing countries, in particular for lemons, oranges, mandarins, tangerines and clementines (FAOSTAT, 2023). Kumquat (*Fortunella*) is a close relative of the *Citrus*, defined as producing the smallest citrus fruit. *Fortunella* was included for several decades within *Citrus* until Swingle (1943) reclassified the genus *Fortunella*, based on morphological and phenological characteristics. The 'short oblong to round' kumquat Meiwa (*F. crassifolia* Swingle), 'oval' kumquat Nagami (*F. margarita* (Lour.) Swing-

le) and 'round' kumquat Marumi (*F. japonica* (Thunb.) Swingle) are the most widely cultivated *Fortunella* species, characterized by small, flavourful, and brilliant fruit with agronomic traits that differ from other citrus taxa (Zhu *et al*., 2022).

In Europe, kumquat has been grown in the Mediterranean regions for its ornamental value and applications in pharmaceutical, sanitary, cosmetic, agriculture and food industries. Kumquat fruit are important sources of nutrients and of phytochemicals that can prevent human diseases (Chen *et al*., 2017; Al-Saman *et al*., 2019). Italy is the European leader in the production of ornamental citrus plants, with Sicily accounting for more than 90% of Italian production. The 'oval' kumquat is the most important ornamental citrus plant, after lemon (*Citrus limon* (L.) Burm f.) and calamondin (*Citrus madurensis* Lour.) (Sottile *et al*., 2019).

The increasing distribution and economic importance of kumquat are threatened by several fungal diseases, which are major causes of preharvest production losses. *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. causes trunk canker, dieback and gummosis, and some *Fusarium* spp. cause shoot and branch canker and tree decline in China and Taiwan (Ko *et al*., 2004; Zhu *et al*., 2013; Gui *et al*., 2020). A survey in major citrus-producing countries showed *Diaporthe novem* J.M. Santos, Vrandečić & A.J.L. Phillips and *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. were associated with kumquat twig dieback, whereas *C. karsti* You L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai (as '*karstii*') (Yang *et al*., 2011) was associated with leaf lesions, although no conclusion was drawn on its pathogenicity role (Huang *et al*., 2013; Guarnaccia *et al*., 2017).

During field surveys in Southern Italy, previously unobserved and severe symptoms of twig and branch dieback and gummosis were found. These were similar to those reported as a new disease in California in two kumquat orchards (Mayorquin *et al*., 2019; Camilletti *et al*., 2022), on other hosts (*C. sinensis* 'Cara Cara' and 'Fisher', *C. reticulata* 'Clemenules', *C. reticulata* '4B'). *Colletotrichum* includes important plant pathogens that are widespread (Timmer *et al*., 2000; Lima *et al*., 2011; Dean *et al*., 2012; Vitale *et al*., 2020), and is a pathogen genus which also includes species of endophytes, epiphytes or saprobes that can switch behaviour to pathogenic in host plants growing in stress conditions (Crous *et al*., 2016). *Colletotrichum* spp. infect a wide range of ornamental plants and tropical, subtropical and temperate fruit crops (Bernstein *et al*., 1995; Freeman and Shabi, 1996; Freeman *et al*., 1998; Polizzi *et al*., 2011; Aiello *et al*., 2015; Ismail *et al*., 2015; Guarnaccia *et al*., 2017, 2019, 2021; Vitale *et al*., 2021). Numerous species of *Colletotrichum* are recognized to affect citrus and allied genera (*Atlantia*, *Fortunella*, *Microcitrus*, *Murraya*, *Poncirus*), and are included in four species complexes (SCs), namely gloeosporioides SC (Cannon *et al*., 2008; Phoulivong *et al*., 2011; Weir *et al*., 2012), acutatum SC (Marcelino *et al*., 2008; Shivas and Tan, 2009; Damm *et al*., 2012b; Baroncelli *et al*., 2015), boninense SC (Moriwaki *et al*., 2003; Yang *et al*., 2009; Damm *et al*., 2012a) and truncatum SC (Damm *et al*., 2009; Cannon *et al*., 2012). These pathogenic fungi are well-known to cause anthracnose, post bloom fruit drop, tear stain, stem-end rot, and withered twig tips on several citrus hosts (Brown *et al*., 1996; Timmer *et al*., 2000; Peres *et al*., 2008; Lima *et al*., 2011; McGovern *et al*., 2012; Riolo *et al*., 2021), and losses of marketable fruit (Aiello *et al*., 2015; Ramos *et al*., 2016; Rhaiem and Taylor, 2016). *Colletotrichum karsti* is the most common and geographically diverse species in the boninense SC (Damm *et al*., 2012b) which was reported in many countries affecting several tree hosts, including citrus (Aiello *et al*., 2015; Ramos *et al*., 2016; Taheri *et al*., 2016; Mayorquin *et al*., 2019; Uysal and Kurt, 2019; Riolo *et al*., 2021; Vitale *et al*., 2021; Wang *et al*., 2021; Camilletti *et al*., 2022; Nodet *et al*., 2023).

The objectives of the present study were: (i) to identify the fungal species associated with twig and branch dieback and gummosis of kumquat in Southern Italy, using morphological characteristics and multi-locus phylogenetic analyses; and (ii) to assess the pathogenicity and aggressiveness of representative isolates obtained from surveyed kumquat plants.

MATERIALS AND METHODS

Field surveys, sampling and fungal isolation

A 2-year survey was conducted in two commercial orchards of kumquat (*F. margarita*) trees that were showing severe dieback and gummosis. The orchards were located in Giarre (approx. 5,000 8-year-old trees) and Mascali (approx. 1,500 22-year-old trees), in Eastern Sicily, Italy. During this period, orchard management maintained favourable and balanced water and nutritional status, and a summer pruning was carried out on symptomatic trees at the end of the first year to remove infected twigs and branches and reduce fungal inoculum. Surveys were conducted from March to July in 2022 and from January to April in 2023. Disease incidence and symptom severity were assessed on the trees at the end of each of these surveyed periods. During 2022, symptomatic twig and branch samples were collected. Forty twigs and branches from each diseased

tree were randomly collected into plastic bags and transferred to the laboratory of Plant Pathology at the Dipartimento di Agricoltura, Alimentazione e Ambiente, University of Catania, for isolation and further analyses. A total of 200 twig fragments (each 5 × 5 mm) were surface sterilized in sodium hypochlorite solution (1.2%) for 60 s and rinsed once in sterilised water. The fragments were dried in sterilised tissue paper, placed onto potato dextrose agar (PDA, Lickson) amended with 100 mg L^{-1} of streptomycin sulfate (Sigma-Aldrich) (PDAS) to prevent bacterial growth, and then incubated in the dark at 25 ± 1°C for 3–4 d. Fungal colonies growing from tissue fragments were transferred onto fresh PDA, and hyphal tips of emerging fungi were sub-cultured onto PDA. Resulting isolates were stored in the laboratory fungal collection.

DNA extraction, PCR amplification and sequencing

Nine fungal isolates (KUM1, KUM6, KUM8, KUM9, KUM10, KUM12, KUM13, KUM14, KUM61) were grown on PDA for 7 d at 25°C. Resulting mycelium of each isolate was harvested with a sterile scalpel, and the genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation), according to the manufacturer's protocol. DNA amplification and sequencing of a combined dataset of loci were carried out to achieve species identification. The partial glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*) was amplified with primers GDF1-GDR1 (Guerber *et al*., 2003). The primers T1 (Glass and Donaldson, 1995) and Bt-2b (Carbone and Kohn, 1999) were used to amplify part of the β-tubulin gene (*tub2*). The partial γ-actin gene (*act*) was amplified using primers ACT-512F and ACT-783R (Carbone and Kohn, 1999). The PCR amplification mixtures and cycling conditions adopted for all three loci were as described by Guarnaccia *et al.* (2017). An amount of 5 μL of PCR product for each PCR reaction was used to assess PCR amplification, by electrophoresis at 100 V on 1% agarose (VWR Life Science AMRESCO® biochemicals) gels stained with GelRedTM. PCR products were sequenced by Eurofins Genomics Service. The DNA sequences were analysed using the program Geneious v. 11.1.5.

Phylogenetic analyses

The sequences obtained in this study were compared with NCBIs GenBank nucleotide database through the standard nucleotide Basic Local Alignment Search Tool (BLAST), to determine the closest species for a taxonomic framework of the studied isolates. Different genomic regions, including new obtained sequences and reference sequences downloaded from GenBank, were initially aligned using the MAFFT v. 7 online server (http: //mafft.cbrc.jp/alignment/server/index. html) (Katoh and Standley, 2013), and were then manually adjusted in MEGA v. 7 (Kumar *et al*., 2016).

Phylogenetic analyses were first carried out individually for each locus (data not shown), and then as multi-locus analyses of three concatenated loci. Additional reference sequences were selected based on recent studies of the genus *Colletotrichum* (Guarnaccia *et al*., 2017; Uysal *et al.,* 2022). Phylogenic analyses were developed based on Maximum Parsimony (MP) for the individual loci, and based on MP and Bayesian Inference (BI) for multi-locus analyses. For BI analyses, the best evolutionary model was estimated using MrModeltest v. 2.3 (Nylander, 2004) for each partition. MrBayes v. 3.2.5 (Ronquist *et al*., 2012) was used to generate the best phylogenetic tree, based on optimal setting criteria for each partition through the Markov Chain Monte Carlo (MCMC) method. The MCMC analyses used four chains and started from a random tree topology. Pre-burn and heating parameters were set, respectively, to 0.25 and 0.2. The trees were sampled every 1000 generations, and analyses ended when the average standard deviation of split frequencies was less than 0.01. Multi-locus analyses based on MP was carried out with Phylogenetic Analyses Using Parsimony (PAUP) v. 4.0b10. Phylogenetic relationships were estimated by heuristic searches with 100 random additional sequences. Tree bisection reconnection (TBR) was used with branch swapping option on "best trees", with all characters weighted equally and gaps processed as fifth base. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC) were calculated to estimated parsimony. Bootstrap analyses were based upon 1000 replications, and resulting trees were visualized with FigTree version 1.6.6. Sequences generated in this study were deposited in GenBank (Table 1).

Assessments of isolate aggressiveness on detached apple fruit

Apple fruit (*Malus domestica* (Suckow) Borkh.) 'Golden Delicious', known to be highly susceptible to *Colletotrichum* diseases (Freeman *et al*., 1998; Lakshmi *et al*., 2011), were used to assess aggressiveness among the selected (above) *C. karsti* isolates, using methods of Chen *et al*. (2022). Healthy and unwounded apple fruit obtained from a commercial market were washed under running tap water, surface sterilized with 70%

Table 1. Collection details and GenBank accession numbers of *Colletotrichum* isolates included in this study.

Species	Culture code[a]	Host	Organ	Locality	Collector	GenBank No.[b]		
						gapdh	*act*	*tub2*
Colletotrichum annellatum	**CBS 129826**	*Hevea brasiliensis*	Leaf	Colombia	L. Maria Hoyos-Carvajal and O. Castro	JQ005309	JQ005570	JQ005656
C. beeveri	**CBS 128527**	*Brachyglottis repanda*	Leaf	New Zealand	R.E. Beever	JQ005258	JQ005519	JQ005605
C. boninense	**CBS 123755**	*Crinum asiaticum 'Sinicum'*	-	Japan	T. Sato	JQ005240	JQ005501	JQ005588
C. brasiliense	**CBS 128501**	*Passiflora edulis*	Fruit	Brazil	N. Massola and H.J. Tozze	JQ005322	JQ005583	JQ005669
C. brassicicola	**CBS 101059**	*Brassica oleracea 'Gemmifera'*	Leaf	New Zealand	B. Thrupp	JQ005259	JQ005520	JQ005606
C. catinaense	**CBS 142417**	*Citrus reticulata*	Leaf	Italy	V. Guarnaccia	KY856224	KY855971	KY856482
C. citricola	**CBS 134228**	*Citrus unchiu*	-	China	F.Huang	KC293736	KC293616	KC293656
C. colombiense	**CBS 129818**	*Passiflora edulis*	Leaf	Colombia	L. Maria Hoyos-Carvajal and D. Riascos	JQ005261	JQ005522	JQ005608
C constrictum	**CBS 128504**	*Citrus limon*	Fruit	New Zealand	P.R. Johnston	JQ005325	JQ005586	JQ005672
C. cymbidiicola	**IMI 347923**	*Cymbidium* sp.	Leaf	Australia	-	JQ005253	JQ005514	JQ005600
C. dacrycarpi	**CBS 130241**	*Dacrycarpus dacrydioides*	Leaf	New Zealand	G.Caroll	JQ005323	JQ005584	JQ005670
C. gloeosporioides	**CBS 112999**	*Citrus sinensis*	-	Italy	-	JQ005239	JQ005500	JQ005587
C. hippeastri	**CBS 125376**	*Hippeastrum vittatum*	Leaf	China	Y.L. Yang	JQ005318	JQ005579	JQ005665
Colletotrichum karsti	CBS 126532	*Citrus* sp.	-	South Africa	-	JQ005296	JQ005557	JQ005643
	CBS 129833	*Musa* sp.	-	Mexico	-	JQ005262	JQ005523	JQ005609
	CBS 129829	*Gossypium hirsutum*	-	Germany	-	JQ005276	JQ005537	JQ005623
	CBS 128551	*Citrus* sp.	-	New Zeland	-	JQ005295	JQ005556	JQ005642
	CPC 27853	*Citrus sinensis*	Fruit	Catania, Italy	-	KY856285	KY856034	KY856543
	CBS 134226	*Citrus limon*	-	China	L. Fang	KC293730	KC293610	KC293650
	CPC 27845	*Citrus sinensis*	Twigs	Catania, Italy	-	KY856284	KY856033	KY856542
	CPC 31139	*Citrus sinensis*	Leaf	Catania, Italy	-	KY856291	KY856040	KY856549
	CPC 31143	*Citrus sinensis*	Twigs	Zurrieq, Malta	-	KY856292	KY856041	KY856550
	CPC 26375	*Citrus paradisi*	Twigs	Catania, Italy	-	KY856277	KY856026	KY856535
	CPC 27077	*Citrus reticulaya novae*	Twigs	Almeria, Spain	-	KY856282	KY856031	KY856540
	CPC 28065	*Citrus limon*	Leaf	Castello, Spain	-	KY856289	KY856038	KY856547
	KUM1	*Fortunella margarita*	Twigs	Giarre, Italy	G. Polizzi	*OR031116*	*OR031125*	*OR001840*
	KUM6	*Fortunella margarita*	Twigs	Giarre, Italy	G. Polizzi	*OR031117*	*OR031126*	*OR001841*
	KUM8	*Fortunella margarita*	Branch	Giarre, Italy	G. Polizzi	*OR031118*	*OR031127*	*OR001842*
	KUM9	*Fortunella margarita*	Twigs	Mascali, Italy	G. Polizzi	*OR031119*	*OR031128*	*OR001843*
	KUM10	*Fortunella margarita*	Twigs	Mascali, Italy	G. Polizzi	*OR031120*	*OR031129*	*OR001844*
	KUM12	*Fortunella margarita*	Twigs	Mascali, Italy	G. Polizzi	*OR031121*	*OR031130*	*OR001845*
	KUM13	*Fortunella margarita*	Branch	Mascali, Italy	G. Polizzi	*OR031122*	*OR031131*	*OR001846*
	KUM14	*Fortunella margarita*	Branch	Mascali, Italy	G. Polizzi	*OR031123*	*OR031132*	*OR001847*
	KUM61	*Fortunella margarita*	Branch	Giarre, Italy	G. Polizzi	*OR031124*	*OR031133*	*OR001848*

(Continued)

Table 1. (Continued).

Species	Culture code[a]	Host	Organ	Locality	Collector	GenBank No.[b]		
						gapdh	*act*	*tub2*
C. limonicola	**CBS 142410, CPC 31141**	*Citrus limon*	Twig	Malta	V. Guarnaccia	KY856296	KY856045	KY856554
C. novae-zelandiae	**CBS 128505**	*Capsicum annuum*	Fruit	New Zealand	P.R. Johnston	JQ005315	JQ005576	JQ005662
C. oncidii	**CBS 129828**	*Oncidium* sp.	Leaf	Germany	U. Damm	JQ005256	JQ005517	JQ005603
C. parsonsiae	**CBS 128525**	*Parsonsia capsularis*	Leaf	New Zealand	B. Weir and G. Carroll	JQ005320	JQ005581	JQ005667
C. petchiii	**CBS 378.94**	*Dracaena marginata*	Leaf	-	P. Di Lenna	JQ005310	JQ005571	JQ005657
C. phyllanthi	**CBS 175.67**	*Phyllanthus acidus*	-	India	H. Surendranath Pai	JQ005308	JQ005569	JQ005655
C. torulosum	**CBS 128544**	*Solanum melongena*	-	New Zealand	B. Weir and P.R. Johnston	JQ005251	JQ005512	JQ005598
Monilochaetes infuscans	**CBS 869.96**	*Ipomea batatas*	-	South Africa	I. Rong	JX546612	JQ005843	JQ005864

[a] CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CPC: Culture collection of P.W. Crous, housed at the Westerdijk Institute; IMI: Culture collection of CABI Europe UK Centre, Egham, UK; KUM: Dipartimento di Agricoltura, Alimentazione e Ambiente, University of Catania, Italy.
[b] *gapdh*: glyceraldehyde-3-phosphate dehydrogenase gene; *act*: actin gene; *tub2*: beta-tubulin gene; *act*: actin gene. Sequences generated in this study are indicated in italics.

ethanol solution using tissue paper, and then air dried on a laboratory bench. Two wounds per fruit (three fruit for each isolate) were made at the widest part, with equal distance between them, using a sterile needle. A conidium suspension was produced for each *C. karsti* isolate, that was previously grown on PDA for 15 d at 25 ± 1°C. An aliquot of sterile distilled water was added to each culture plate, and the mycelium was gently rubbed with a sterile loop, The resulting suspension was filtered through a triple layer of cheesecloth, and conidium suspension was adjusted to 10^5 conidia mL^{-1}, as assessed with a microscope slide haemocytometer. Each fruit was then inoculated by pipetting a 20 µL drop of a conidium suspension onto the wound. Inoculation controls consisted of apple fruit inoculated with distilled water. Fruits were placed into 30 × 12 × 8 cm clean plastic boxes, each containing 200 mL of sterile water to maintain high humidity. The boxes were then covered with plastic film and incubated at 25 ± 1°C with a 12 h photoperiod. Eight days after inoculation (DAI), disease incidence (DI) was evaluated by counting the number of symptomatic inoculation points, and symptoms severity (SS) was determined by measuring two longitudinal diameters (cm) of each lesion. Mean lesion diameter data were recorded, and were statistically analysed (Statistix 10: Analytical Software 2013) using analysis of variance (ANOVA). Mean differences were compared according to Tukey's honestly significant difference (HSD) test at $P < 0.05$.

Pathogenicity tests on kumquat plants

Pathogenicity tests were carried out using three representative isolates (KUM1, KUM6, KUM8) that differed in aggressiveness on apple fruit. The isolates were inoculated onto healthy 2-year-old kumquat plants grafted to volkamerian lemon (*C. volkameriana* Ten. & Pasq.) rootstock. Two inoculation methods were used. In the first experiment, wounds were made by pruning a 6 cm-length twig tips, to reproduce wind damage on plants. Inoculations were carried out by spraying conidium suspension of each *C. karsti* isolate onto the wounds. In the second experiment, twigs were surface disinfected with a 70% ethanol solution, and each wounded by removing a piece of bark (4 × 4 mm) with a sterile scalpel to expose the cambium. Mycelium plugs (4 mm diam.) were taken from the edges of 30-d-old cultures of *C. karsti* grown on PDA, and were placed into the twig wounds (Mayorquin *et al.*, 2019). Inoculated twigs

were covered with Parafilm® (Pechney Plastic Packaging Inc.) to prevent drying. Experimental controls consisted of plant wounds inoculated with PDA plugs. Three plants per isolate (nine twigs per plant) were used in each experiment. All the plants were then transferred into a growth chamber at 25 ± 1°C with a 12-h photoperiod, and were regularly watered. After 30 d, DI was determined by counting the number of symptomatic twigs. To assess fulfilment of Koch's postulates, small pieces of tissue were taken from the dieback bases, then surface sterilised in sodium hypochlorite solution (1.2%) for 60 s, rinsed once in sterilised water, and then plated onto PDA amended with 100 mg L^{-1} of streptomycin sulfate. Emerging fungal colonies were recorded, as described (above).

RESULTS

Field surveys, climate data and fungal isolations

In the two surveyed kumquat orchards, symptoms of dieback were found affecting entire tree canopies (general dieback) (Figure 1, a and b), or a few twigs and branches (sectoral dieback) (Figure 1, c and d). Canopy thinning and defoliation were also observed, although in several cases the leaves did not drop but remained on the twigs and rapidly dried, that ensured the physiological abscission (Figure 1). Dieback on twigs appeared as brown to chocolate-brown clearly-shaped lesions (Figure 2, c and d). Sometimes, abundant typical *Colletotrichum* acervuli were produced on the surfaces of the dead host tissues (Figure 2 e). Generally, symptoms of gummosis also appeared below the twig lesions, as common host responses plants to stress, such as wounding and/or pathogen infection (Figure 2, a and b). Field observations during July 2022 indicated that incidence of the disease differed with different tree age.

Disease incidence based on the number of plants with dieback and gummosis symptoms was approx. 5% of trees in the 22-year-old orchard and 55% of the trees in the 8-year-old orchard. Conversely, symptom severity was greatest in the 8-year-old orchard, with 65 to 70% of young trees each with dieback of one to five twigs on the canopy tops, and with lesions smaller than 10 cm long. Only 20 to 25% of trees in the 8-year-old orchard exhibited dieback of branches, with lesions varying from 10 to 60 cm long. In contrast, symptomatic trees in the 22-year-old orchard showed greater incidence (75 to 80%) of branch dieback, that reached lengths of 50 to 60 cm, and 15 to 20% of the plants had a few apical twigs with lesions smaller than 10 cm long. In both orchards, sporadic dieback of entire canopies was observed.

Since March to April 2023, twig dieback was occasionally observed in the upper tree canopies mainly that were exposed to wind in both of the orchards, with mean disease incidence less than 1%. Adverse mean meteorological conditions, including sudden temperature decreases followed by strong winds occurred in January 2022 before the development of symptoms. In detail, very low temperatures (daily minimum air temperature -2 to +3°C) and strong winds occurred. In contrast, low wind speed events and temperatures that never below 3°C were recorded in January 2023.

Fungal isolates recovered from symptomatic twigs all had the same cultural characteristics. These included production of pale to white mycelium with orange conidial masses in the colony centres, and having pale orange on the reverse sides. All isolates recovered from infected samples were identified as *Colletotrichum*-like, according to the morphological and cultural features described by Damm *et al*. (2012b). Among these, 40 representative isolates recovered from the Giarre orchard, and 25 isolates from the Mascali orchard, were morphologically identified and stored in the collection of Dipartimento di Agricoltura, Alimentazione e Ambiente, sez. Patologia Vegetale, University of Catania. Nine of these isolates were selected for molecular analyses and pathogenicity tests.

Phylogenetic analyses

Three single alignments representing each of the analysed genes (*gapdh, act, tub2*), and one alignment of the three combined genes, were analysed. The alignments produced topologically similar trees. The combined species phylogeny of the *Colletotrichum* isolates consisted of 42 sequences, including the outgroup *Monilochaetes infuscans* (CBS 869.96). The multi-locus phylogenetic analysis included a total of 961 characters (*gapdh*:1-199, *act*: 204-450, *tub2*: 455-961). A total of 285 characters were parsimony-informative, 283 were variable and parsimony uninformative, and 385 were constant. A maximum of 1000 equally most parsimonious trees were saved (Tree length = 1081, CI = 0.804, RI = 0.820 and RC = 0.660). Bootstrap support values obtained with the parsimony analyses are showed on the Bayesian phylogenetic tree (Figure 3). For the Bayesian analyses, MrModeltest suggested dirichlet state frequency distributions for *act* and dirichlet state frequency and fixed state frequency for *gapdh* and *tub2*. As recommended by MrModeltest, the following models were used: K80 + G and KHY + G for *gapdh*, HKY + G for *act* and K80 + G and HKY + I for *tub2*. In the Bayesian analyses, the partial *gapdh* gene had 150 unique site

Figure 1. Symptoms of dieback caused by *Colletotrichum karsti* on kumquat (*Fortunella margarita*) trees; a and b, severe dieback symptoms of entire tree canopies, where leaves remain attached to the twigs; c, dieback of a few branches; d, apical twigs with defoliation.

Figure 2. Symptoms of Colletotrichum dieback on kumquat (*Fortunella margarita*). a and b, gummosis and brown to chocolate-brown lesions on twigs; c, brown internal discolouration of twigs; d, detail of clearly-shape twig lesions; e, typical *Colletotrichum* acervuli on the surface of a dead host branch.

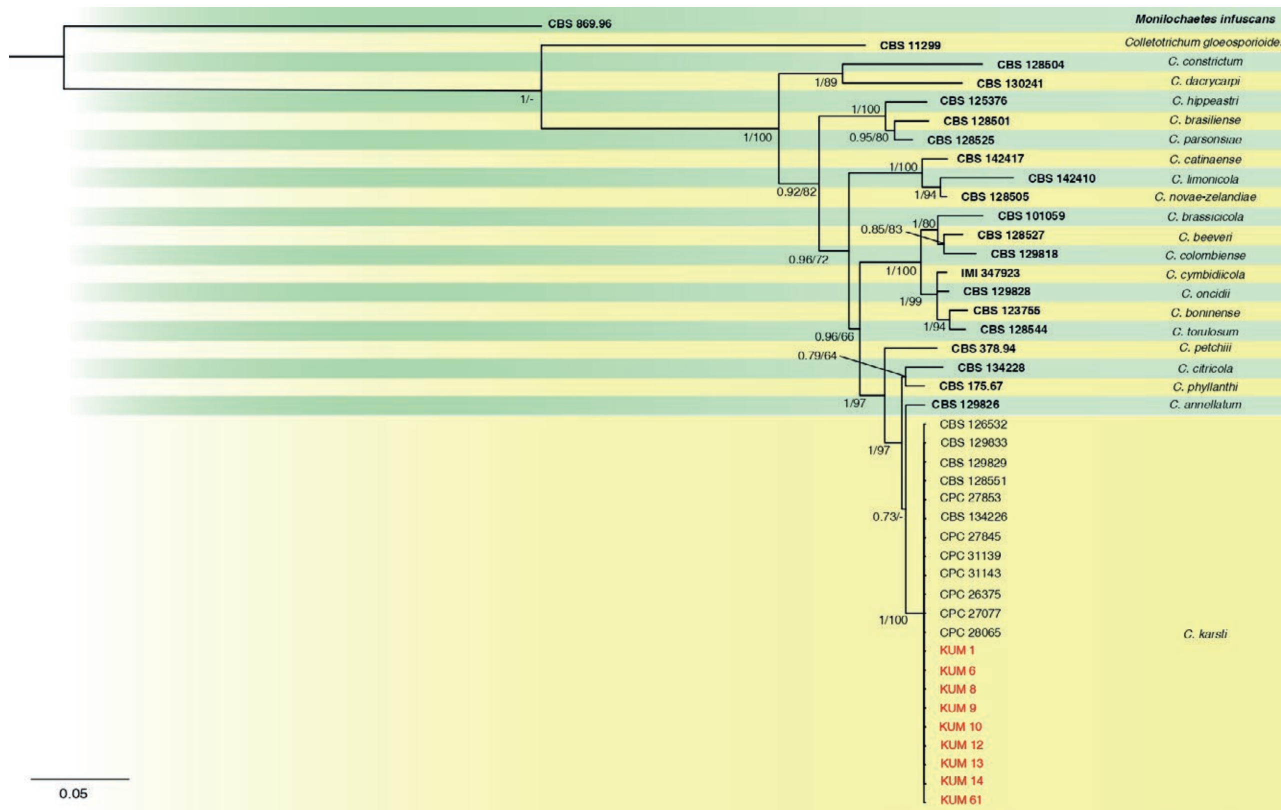

Figure 3. Consensus phylogram resulting from BI of the combined *gapdh*, *act* and *tub2* datasets. Bayesian posterior probability values and bootstrap support values are indicated at the nodes. The tree was rooted with *Monilochaetes infuscans* (CBS 869.96). The fungal isolates used in this study are indicated in red font.

patterns, the partial *act* gene had 120, and the partial *tub2* gene had 220. The analysis ran for 160.000 generations, resulting in 322 trees of which 242 trees were used to calculate the posterior probabilities. Considering the combined analyses, the nine isolates clustered with twelve reference strains of *Colletotrichum karsti,* forming a highly supported clade based on bootstrap values (1/100).

Aggressiveness test of isolates on detached apple fruit

All the tested isolates were pathogenic on wounded apple fruit, giving DIs of 100%, and causing the typical bitter rot with abundant conidia produced in mucilaginous orange masses. The rotted lesions appeared after 3 to 4 d and destroyed the entire fruit within 15 to 20 d. Fruit inoculated with PDA plugs remained healthy (Figure 4 f). The results presented in Figure 5 indicated no significant differences in aggressiveness ($P < 0.05$) 8 DAI between *C. karsti* isolates KUM8 (mean lesion diam. = 1.25 cm) (Figure 4 e), KUM9 (0.98 cm), KUM10 (0.88 cm), KUM12 (0.94 cm), KUM13 (1.00 cm), KUM14 (0.87 cm), KUM61 (1.05 cm), with KUM1 (0.69 cm) caused the least mean lesion diameter, and KUM6 (3.66 cm) caused the greatest (Figure 4 d).

Pathogenicity tests on kumquat plants

In the first experiment, no symptoms were observed when conidium suspensions of *C. karsti* isolates were inoculated on partially broken kumquat twigs. In contrast in the second experiment, the isolates inoculated on wounded twigs using mycelium plugs cause twig dieback at 20 DAI. The affected twigs were brown to chocolate-brown, with clearly-shaped lesions extending under the inoculation points (Figure 4, a and b). Typical acervuli of *Colletotrichum*, and gummosis, was also observed near the inoculation sites (Figure 4 c). DI data based on the numbers of symptomatic twigs on the kumquat plants were 15% for plants inoculated with isolate KUM1, 30% from isolate KUM6 and 20% from isolate KUM8. Symptom severity based on lesion lengths produced by the

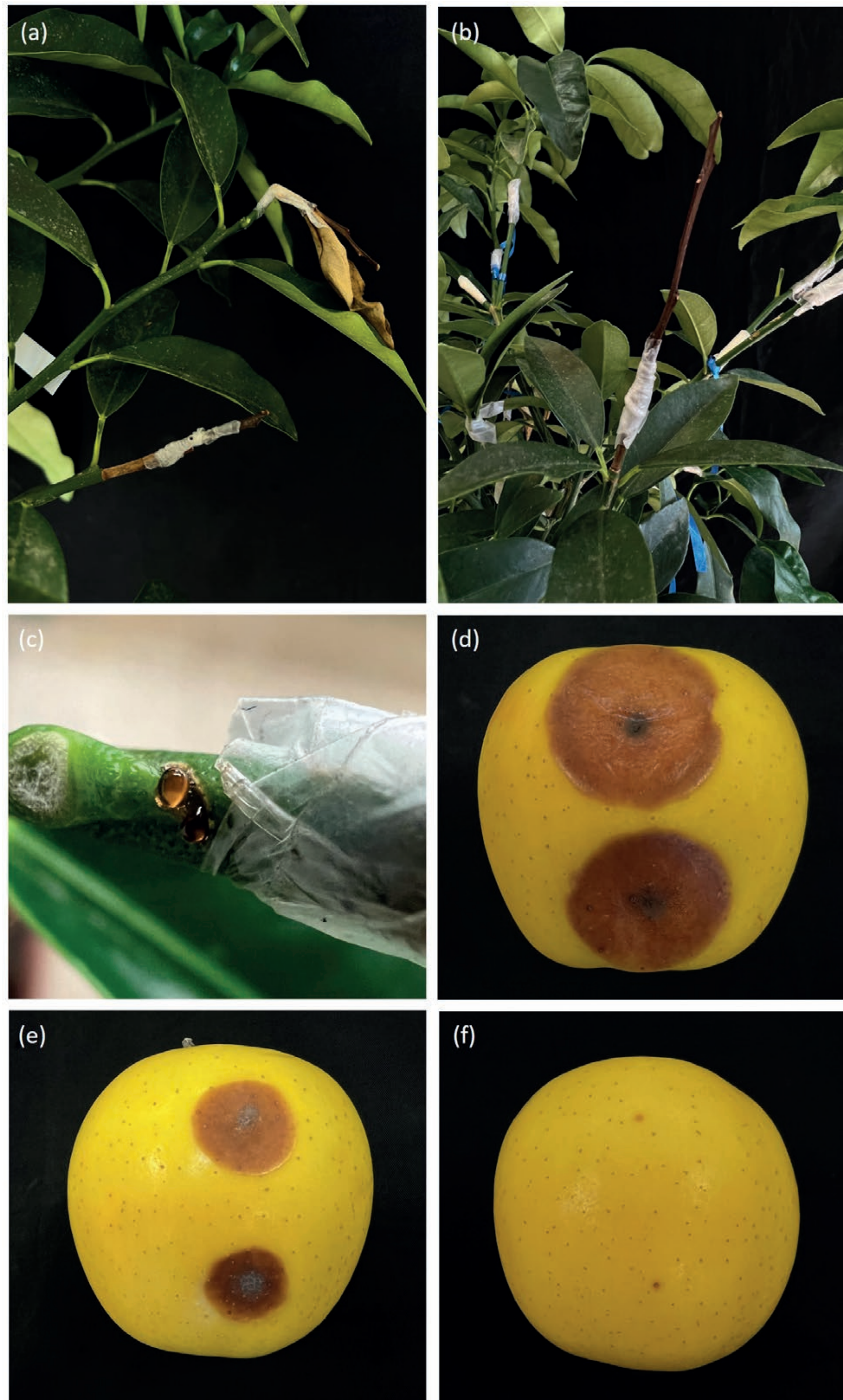

Figure 4. Pathogenicity and aggressivity tests. a and b, symptoms of twig dieback on 2-year-old kumquat plants (*Fortunella margarita*), 20 d after inoculation with mycelium plugs of *Colletotrichum karsti* isolate KUM6. c, detail of gummosis on a kumquat twig below the artificial inoculation point, caused by *C. karsti* isolate KUM6. d and e, necrotic lesions on detached apple fruit 'Golden Delicious' 7 d after inoculations with conidium suspensions of *C. karsti* isolates KUM6 (d) or KUM8 (e). f, a non-inoculated control apple fruit.

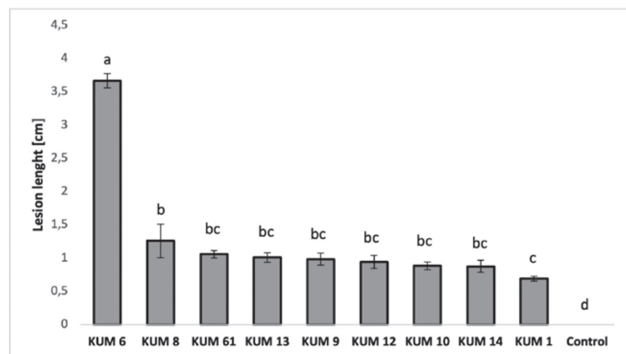

Figure 5. Mean lesion length (cm) resulting from inoculations with different *Colletotrichum karsti* isolates (KUM 6 to KUM 1) onto apple fruit of cultivar 'Golden Delicious' 7 d after inoculations. Different letters above the bars indicate statistically significant differences between the isolates, based on Tukey's honestly significant difference (HSD) test (α = 0.05). The standard deviations of the means are also indicated.

pathogen since could not be assessed because complete withering occurred when the twigs were infected. No disease symptoms were observed in plants used as experimental controls. Colonies of *C. karsti* were recovered from inoculated twigs, whereas no *Colletotrichum* spp. were isolated from the control plants.

DISCUSSION

In this study, the first investigation of twig and branch dieback of kumquat trees (*F. margarita*) in Italy was conducted, thus, molecular analysis and pathogenicity tests were performed demonstrating the identification of *C. karsti*, belonging to the boninense SC, as the causal agent of the reported disease. Host symptoms of twig dieback caused by *Colletotrichum* spp. have been widely reported in other fruit crops, including citrus, but these fungi have not been documented as causing disease on kumquat. Cultivation of allied genera of citrus, including kumquat, has been increasing in Southern Italy. Kumquat has gained significant economic importance due to its ability to tolerate extreme climatic conditions (e.g. freezing temperatures), compared to other citrus species (Morton, 1987; Yang *et al*., 2023), and for its agronomic traits and nutritional properties. Severe symptoms of twig and branch dieback on kumquat trees were reported for the first time in Sicily from March to July of 2022 after low temperatures, windstorms, and rainfall events.

Colletotrichum karsti is a well-known Ascomycete which was described for the first time infecting *Ochidaceae* hosts in China (Yang *et al*., 2011), and then reported elsewhere to cause disease on numerous important plants, including apple (*Malus domestica*) (Velho *et al*., 2019), avocado (*Persea americana* Mill.) (Lima *et al*., 2013), blueberry (*Vaccinium* spp.) (Rios *et al*., 2015), and papaya (*Carica papaya* L.) (Damm *et al*., 2012b). This fungus has also been reported occasionally associated with mango (*Mangifera indica* L.) (Damm *et al*., 2012b) and olive (*Olea europaea* L.) (Schena *et al*., 2014). On citrus hosts, *C. karsti* was first reported by Aiello *et al*. (2015), as causing twig wither tips and anthracnose on sweet orange. More recently, a new disease (twig and branch dieback) caused by *C. karsti* was reported on lemon in Portugal (Ramos *et al*., 2016) and on sweet orange and clementine in California (Mayorquin *et al*., 2019). Mayorquin *et al*. (2019) reported *C. karsti* as a pathogen causing wood canker, but this fungus has not been associated with other known *Botryosphaeriaceae* or *Diatrypaceae* canker and dieback pathogens of citrus (Bezerra *et al*., 2021). Severe twig wither tip, twig and branch dieback and anthracnose symptoms caused by *C. gloeosporioides* and *C. karsti* have been reported on sweet orange (*Citrus sinensis* 'Valencia', 'Navel', 'Tarocco' and other blood orange hosts), lemon (*C. limon* 'Femminello Siracusano 2KR' and 'Zagara bianca'), mandarin (*Citrus* × *clementina* 'Nova', 'Mandalate' and 'Yosemite Gold') and mandarin-like hosts (*C. clementina* × 'Orlando' tangelo, 'Fortune', and *C. clementina* 'Nules' × *C. sinensis* 'Tarocco', 'Mandared'), in Italy (Riolo *et al*., 2021; Vitale *et al*., 2021), Albania (Riolo *et al*., 2021), and Turkey (Uysal and Kurt, 2019; Uysal *et al*., 2022).

In the present study, phylogenetic analyses of selected fungal isolates showed that *C. karsti* was the only species associated with twig dieback of kumquat. *Botryosphaeriaceae* and *Diaporthaceae*, which are generally associated to dieback diseases (Guarnaccia and Crous, 2017; Bezerra *et al*., 2021), were not isolated from symptomatic samples, and *C. gloeosporioides* was not found among *Colletotrichum* isolates. Nevertheless, co-occurrence of the two *Colletotrichum* species is possible on kumquat plants, because of the small number of molecularly characterised isolates. Previous studies evaluating aetiology of citrus twig dieback (Huang *et al*., 2013; Aiello *et al*., 2015; Ramos *et al*., 2016; Mayorquin *et al*., 2019; Riolo *et al*., 2021; Camilletti *et al*., 2022) have shown inconsistent results for the most frequently detected *Colletotrichum* species from diseased plants. The present study results were similar to those of Mayorquin *et al*. (2019) and Camilletti *et al*. (2022), who reported *C. karsti* as the most frequently identified species collected from twig dieback on orange, lemon and mandarin. In contrast, Huang *et al*. (2013), Aiello *et al*. (2015), Ramos *et al*. (2016) and Riolo *et al*. (2021) observed prevalence of *C.*

gloeosporioides associated with dieback diseases. A recent study by Uysal *et al.* (2022) showed that *C. karsti* was common on twigs, branches, and leaves of lemon, while *C. gloeosporioides* predominated in flowers and fruit.

Inconsistencies on composition and distribution of *Colletotrichum* species in commercial orchards may depend on the host susceptibility, environmental conditions, and cultural practices, such as fungicide selection pressure (Leandro *et al.*, 2003; Diéguez-Uribeondo *et al.*, 2011; Moral *et al.*, 2018; Piccirillo *et al.*, 2018; Veloso *et al.*, 2021; Tan *et al.*, 2022). A strong relationship between climatic conditions and Colletotrichum pathosystems has been reported, suggesting that *Colletotrichum* species differ in temperature requirements for conidial germination and appressorium formation (Camilletti *et al.*, 2022). The present study results on aggressiveness of *C. karsti* isolates on apple fruit showed significant differences among some isolates (KUM1, KUM6 and KUM8). Camilletti *et al.* (2022) reported no intraspecific variability in aggressiveness among isolates of *C. karsti* inoculated on navel orange in a Californian orchard. However, several authors have reported that *Colletotrichum* isolates belonging to the same fungal species show variability in aggressiveness when collected from different hosts (Giblin *et al.*, 2010; De Silva *et al.*, 2021). Consequently, although a limited number of isolates was used, the present study indicates that *C. karsti* isolates associated with kumquat dieback may differ in aggressiveness.

The ability of *C. karsti* to efficiently infect plants, thereby exhibiting high aggressiveness, and in comparisons with *C. gloeosporioides*, has been investigated by several authors. *Colletotrichum karsti* was reported to be less aggressive than *C. gloeosporioides* when inoculated on detached sweet orange, lemon and apple fruit in growth chamber experiments, and on sweet orange twigs in field experiments (Aiello *et al.*, 2015; Guarnaccia *et al.*, 2017; Riolo *et al.*, 2021), whereas Mayorquin *et al.* (2019) observed opposite results on clementine plants. The recent study of Camilletti *et al.* (2022) in California assessed a large number of isolates on navel orange, and showed that *C. karsti* was as aggressive as *C. gloeosporioides*. Pathogenicity tests on kumquat plants in the present study showed that *C. karsti* can cause twig dieback and gummosis when inoculated with mycelial plugs (the second inoculation method used in the present study), whereas symptoms were not observed on twigs when they were inoculated by spraying conidium suspensions (first inoculation method).

The difficulty of reproducing field symptoms on kumquat plants in growth chamber conditions could be attributed to environmental effects on epidemiology of *Colletotrichum* infections. Sudden temperature decreases, strong winds and rain occurred before the observation of symptoms in the field in January 2022, and these may have affected the susceptibility and responses of plants to infection, as well as the growth, survival, and spread of *Colletotrichum*, which has been reported to switch to pathogenic behaviour in plants growing in stress conditions (Crous *et al.*, 2016). Nevertheless, the attempt to reproduce stress effects from climatic factors by wounding plants before artificial inoculation of *C. karsti* was not enough to substitute the role of favourable environmental conditions for disease development, as has been observed in other studies (Mayorquin *et al.*, 2019; Riolo *et al.*, 2021).

The present study has identified *C. karsti* as the causal agent of twig and branch dieback of kumquat, and these results highlight the importance of implementing sustainable management strategies for an emerging plant pathogen able to infect an increasing number of plants species. These results are also relevant for future scenarios of increasing climate change that could contribute to favourable conditions for pathogen development and spread in temperate regions.

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Short Notes

Symptomatic, widespread, and inconspicuous: new detection of tomato fruit blotch virus

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Summary. Tomato production is an important part of the Swiss vegetable production with most tomato crops grown in greenhouses. Tomato plants are vulnerable to diseases caused by viruses, which can have significant impacts on crop production. This study reports the first detection of tomato fruit botch virus (ToFBV, *Blunervirus solani*) in Switzerland, from a tomato production site at the southern part of the Ticino region. The symptoms observed indicated presence of a viral pathogen, but tests against the most common tomato viruses were negative. Immunocapture of double-stranded RNA and its subsequent sequencing on a Flongle flowcell (Oxford Nanopore Technologies) identified the presence of ToFBV and southern tomato virus. The genome of the Swiss ToFBV isolate was very similar to that available in GenBank. Datamining of the sequence read archives found the virus in two other countries, with a highly conserved genome. With this study, there are now 12 near-complete genomes of ToFBV available, and the virus is recorded from ten countries. This study underlines the importance of continuous monitoring and research on emerging viruses in tomato production.

Keywords. *Kitaviridae, Blunervirus solani*, Flongle sequencing, dsRNA.

INTRODUCTION:

Tomato production (*Solanum lycopersicum*) is an important part of the Swiss agricultural sector, with more than 40,000 metric tons produced in 2021. Of the production area, 4% was in open fields, and 96% was in greenhouses, with almost 60% using the soilless production systems (Swiss Federal Statistical Office, 2022). Tomato plants are vulnerable to a range of pests and diseases, often depending on climate, geographical location, and production system. These pests and pathogens include insects, nematodes, fungi, oomycetes, bacteria, and viruses (Panno *et al.*, 2021). A significant number of pathogens are well-known, researched, and controlled; however, the industry is also confronted with emerging diseases, many of which are associated

with viruses. In a recent review, Rivarez *et al.* (2021) listed more than 312 virus, satellite virus, satellite RNA or viroid species (in 22 families and 39 genera) associated with tomato.

In the last decade, tomato brown rugose fruit virus (ToBRFV) has rapidly spread across 35 countries, and has had significant impacts on world tomato production (Caruso *et al.,* 2022). ToBRFV was reported in Switzerland in 2021, from soil-grown tomato production in the north-east of the country (Mahillon *et al.,* 2022). Another, lesser-known symptomatic and emerging virus, tomato fruit blotch virus (ToFBV, *Blunervirus solani*), was identified in 2020 in Italy and Australia (Ciuffo *et al.,* 2020). ToFBV is in the family *Kitaviridae*, a group of plant viruses distantly related to nege-like viruses that mainly infect invertebrates (Ramos-González *et al.,* 2022). In the past 2 years, the virus was detected in Spain, Portugal, Brazil, Tunisia and Slovenia (Kitajima *et al.,* 2022; Maachi *et al.,* 2021; Nakasu *et al.,* 2022; Rivarez *et al.,* 2022). The virus was detected from symptomatic tomato plants, and uneven and/or deformed fruits were indicated when the symptoms were precisely described (Ciuffo *et al.,* 2020; Kitajima *et al.,* 2022; Nakasu *et al.,* 2022).

The present study reports the first detection of ToFBV in Switzerland, and extends the probable distribution of this virus through mining of publicly available sequence read archive (SRA) data.

MATERIAL AND METHODS

In August 2022, an inspector from the Ticino Agricultural Advisory Office visited a tomato production site in the south of the Ticino region (the southern tip of Switzerland) in response to a request from the producer. Tomato plants were grown in soil under plastic tunnels. Because of the unusual nature of the symptoms observed, samples were collected for laboratory analysis. Severe symptoms observed on fruits included chlorotic rings and sometimes distortion, and these indicated presence of a viral pathogen (Figure 1). After confirming the absence of tomato brown rugose fruit virus (ToBRFV) using specific RT-qPCR, samples were tested for 16 additional known virus, viroid or phytoplasma pathogens of tomato, using lateral flow devices, ELISA, RT-PCR and RT-qPCR. All of these tests were negative, and no particles were observed using a transmission electron microscope.

A double-stranded RNA (dsRNA) extraction was prepared by immunocapture (Blouin *et al.*, 2016). Approximately 3 g of frozen leaf tissue were ground in liquid nitrogen and added to 11.2 mL of extraction buffer (STE with 0.3% bentonite, 2% PVP, 1.5% SDS and 2% beta-mercaptoethanol). The extract was then added to 8 mL of phenol in a 50 mL tube. The sample was vortexed for 1 min, and then centrifuged for 4 min at 2,200 *g*. From the aqueous phase, 6.4 mL was collected and added to 5.1 mL of isopropanol in a new tube. The sample was kept on ice for 10 min, and total nucleic acids were then precipitated by centrifugation at 17,000 *g* for 20 min at 4°C. The resulting pellet was rinsed twice with 75% ethanol, air dried for 10 min, and then resuspended in 2 mL Tris-buffered saline-tween (TBST, 25 mM Tris, 0.15M NaCl, pH 7.5 + 0.05% Tween). From the 2 mL, 1.5 mL were used for the immunocapture, and the remaining 0.5 mL was then kept in a -20°C freezer. Single-stranded RNA was digested with 187.5 U of RNAse T1 (Thermo Fisher Scientific) for 1 h at 37°C on a thermal mixer.

Immunocapture of the dsRNA was prepared as described by Blouin *et al.* (2016). A total of 10 µL of Protein L magnetic beads (Thermo Fisher Scientific) was washed three times in TBST buffered as per the manufacturer recommendation. A total of 400 µL of monoclonal antibody hybridoma supernatant 2G4 (O'Brien *et al.,* 2015; UniQuest Pty Limited) was added to the beads, and these were then incubated at room temperature for 1 h on a rotary mixer. The beads were then washed three times in TBST, resuspended in TBST and added to the RNAse T1 digested extract. The sample was then incubated 1 h at room temperature on a rotary mixer. The beads were then washed three times with TBST buffer, air dried for a few minutes, and resuspended in 20 µL of ultrapure water. The cDNA was synthetized from the dsRNA after an initial heating at 99°C for 2 min of 9 µL of the resuspended beads with 4 µL of linker primer CGTGGAGACTCTGGNNNNNNNNNNNT at 1 µM. The sample was then immediately placed on ice, and the mix was completed with dNTPs (0.5 mM final), 4 µL of 5x Buffer RT, 1 µL of ultrapure water, 20 U of RNAsin® Ribonuclease inhibitor (Promega Corporation), and 100 U of Maxima H Minus (Thermo Fisher Scientific). The sample was kept on ice for 15 min, then incubated for 10 min at 25°C, followed by 30 min at 50°C, and the enzymes were deactivated with a final step of 5 min at 85°C. Remaining RNA was removed by adding 0.75 µL of RNAse A (20 mg mL^{-1}) and incubated for 15 min at 22°C and 2 min at 85°C.

The cDNA was then purified with the AMPure XP (Beckman Coulter) following the manufacturer recommendations, and eluted in 30 µL. PCR was then carried out with the LongAmp Taq 2× Master Mix (New England Biolabs), with 5 µL cDNA, 5 µL MID primer (multiplex identifier) AAGGTAGAAGCGTGGAGA-

CTCTGG, and 10 μL of mastermix. The initial cycle was 95°C for 5 min, 65°C for 30 min and 75°C for 1 min, then followed by 30 cycles each of 94°C for 30 sec, 50°C for 30 sec and 72°C for 3 min, and a final extension of 10 min at 72°C. The sample was then loaded on an agarose gel to visualize the band size. The PCR was cleaned with the AMPure XP (Beckman Coulter) following the manufacturer recommendations and eluted in 20 μL. A DNA concentration of 55 ng μL^{-1} was measured by Qubit Fluorometric Quantification (Thermo Fisher Scientific), and the median size of the DNA amplicon was estimated to be 1,200 bp from the agarose gel. A total of 0.65 μL of the cleaned PCR product was used in the ligation to a concentration of 50 fmol. The ligation sequencing amplicons (kit SQK-LSK110, Oxford Nanopore Technologies) was used as recommended by the manufacturer. The sample was loaded on a Flongle (68 active pores at start) for a 24 h run, and sequenced alongside with another extract from a different plant (*Vitis vinifera* L.), as part of a different experiment but following the same protocol with a different linker and MID.

Virus sequences were recovered from the data by mapping against a reference database of plant viruses using Minimap2 (2.24) plugin (Li, 2021) on Geneious Prime (v2022.0.2 https://www.geneious.com/). The presence of ToFBV was confirmed by RT-PCR using the primers of Nakasu *et al.* (2022), and a gap in the RNA3 was filled with the following primers; ToFBV-RNA3_1823 F (TCTTCGGTCTGCTCGTGATG) and ToFBV-RNA3_2777 R (CGAAACAGAGACCCGTC-CAA). Amplicons were Sanger sequenced. Genome reconstruction was carried out using Geneious Prime.

Datamining was carried out to find additional evidence of the virus, using Serratus (https://serratus.io/) to screen publicly available SRAs deposited before January 2021 (Edgar *et al.,* 2022). The positive SRA files were imported in the Galaxy platform (usegalaxy.org, The Galaxy Community, 2022), where the genomes of ToFBV were reconstructed using a combination of *de novo* sequencing with rnaviralSPAdes (Galaxy Version 3.15.4; Prjibelski *et al*., 2014; Antipov *et al*., 2015; Vasilinetc *et al*., 2015) and reference mapping (Bowtie2 version 2.5.0; Langmead *et al.,* 2009).

RESULTS AND DISCUSSION

From the Flongle sequencing run, a total of 160,241 reads were recovered (167 Mb) with a N50 of 895 nt. Five viruses and one viroid were retrieved from the grapevine sample. Two non-grapevine viruses were detected from the tomato sample, and these were : southern tomato virus (*Amalgavirus lycopersici*) with 18,835 mapped reads, 100% horizontal coverage and 99.97% similarity to isolate Thailand LC487710; and ToFBV, where a total of 38,090 reads mapped the four RNAs with horizontal coverage greater than 98% (Table 1).

Southern tomato virus is a seed-borne virus that is most often asymptomatic. It is widespread, as shown by the 129 accessions deposited in GenBank to date from 25 countries, including Switzerland (Sabanadzovic *et al.,* 2009; Turco *et al.,* 2018).

The four almost complete polyadenylated segments of ToFBV shared the same structure as the other members of the species. All segments recovered were contiguous (only the extremities missing), with the exception of the RNA 3 where a short gap was observed near the 3' end. This gap was completed by RT-PCR. The largest RNA fragment (5,606 nt) encodes a large polyprotein with a methyl-transferase and a helicase recognized domains, the second RNA (3580 nt) encodes a polyprotein with a viral helicase and the RNA-dependent RNA Polymerase (RdRP) domains. The RNA 3 (2755 nt) contains five putative ORFs including one coding the SP24 superfamily motif (putative virion membrane proteins). The RNA 4 (1924 nts) contains two putative ORFs including one coding a putative movement protein.

The four RNA segments recovered were deposited in GenBank (OQ849577- OQ849580). Blast analyses confirmed the close relationship among the virus isolates available. The four Swiss RNA segments closest matches

Table 1. Molecular features of the four RNA segments of tomato fruit blotch virus detected in Switzerland and closest isolate.

RNA	Length (nt)	Horizontal coverage (%)	Read mapped	Nucleotide identity by nBlast[a] (%)	Closest accession by nBlast (Country)
RNA1 (OQ849577)	5,606	99.3	521	99.42	MZ401001 (Tunisia)
RNA2 (OQ849578)	3,580	99.3	221	99.36	MW546268.1 (Brazil)
RNA3 (OQ849579)	2,755	99.1	10,799	98.44	OL472085.1 (Slovenia)
RNA4 (OQ849580)	1,924	98.4	26,549	99.53	MK517480.2 (Italia)

[a] Query coverage >98% and e-value = 0.

Figure 1. Tomato fruit symptoms observed from tomato fruit blotch virus infested site in Ticino, Switzerland.

were from four different isolates, with the percentage identity greater than 98% (Table 1).

ToFBV was also detected by datamining using Serratus (https://serratus.io/). The palmID analysis of viral RdRP identified palmprint with 100% homology to ToFBV from two different bioprojects: one SRA from the bioproject PRJNA626066 "Metaviromic analysis of South Africa sweet potato" (SRR11566106), and three SRAs from the PRJNA491201 "Tomato fruit inoculated with various fungal pathogens" (SRR7841169; SRR7841291; SRR7841300 tomato inoculated with *Fusarium acuminatum* or *Rhizopus stolonifer*) (Petrasch *et al.,* 2019). Nucleotide sequence data reported are available in the Third-Party Annotation Section of the DDBJ/ENA/GenBank databases, under the accession numbers TPA: BK063407 to BK063422. All the genomes showed high degrees of homology, with >95% nucleic acids identity on the four ToFBV RNA segments.

With the addition of the Swiss isolate and the four isolates obtained from the SRAs, there are now 12 near-complete genomes of ToFBV available. Although all publications of ToFBV to date have reported tomato as the only host, GenBank accessions of the Tunisian isolate indicate that the virus was sequenced from potato (*Solanum tuberosum* L.). Similarly, the South African isolate was reconstructed from SRA originating from sweet potato (*Ipomoea batatas*). These two non-tomato hosts should be confirmed by a complementary assay to validate the new host-virus associations. Nevertheless, it is notable that this virus was first described 3 years ago, but is now present, with a highly conserved genome, in ten countries across five continents (Australasia, Eurasia, Africa, North and South America)

This wide distribution resembles that observed for Physostegia chlorotic mottle virus (PhCMoV, *Alphanucleorhabdovirus physostegiae*) first reported in 2018 and then rapidly reported in several countries (Temple *et al.,* 2022). As with ToFBV, most of the PhCMoV sequences in GenBank are almost complete genomes. As diagnostic tools are not yet available, high-throughput sequencing (HTS) is being used to determine the unidentified viral pathogens. PhCMoV also has a highly conserved genome, and the symptoms on the tomato fruits could be mistaken. However, the occurrence of PhCMoV has been, to date, restricted to Europe (Temple *et al.,* 2022).

As observed with PhCMoV, ToFBV can remain in the environment undetected for some time, as suggested by the 10-year-old isolates sequenced from Italy (Ciuffo *et al.,* 2020) and Tunisia. The rarity and sporadic detection of ToFBV contrasts with its worldwide distribution and its conserved genome, although this has also been observed with some other members of the *Kitaviridae* (e.g., *Cileviruses*). The improved surveillance and detection, for example with the rise of HTS technology to detect plant viruses, can partially explain the widespread detections, although this technology has been applied in many laboratories for most of the last decade. Global trade could also explain how the virus and its suspected mite vector are transported across continents (Ramos-González *et al*., 2023). The conserved genome of the virus is also probably the result of better and longer adaptation to its vector than to its host plants. Kitavirids arise from interkingdom virus transfer (Doljan *et al*., 2020). They are the only plant viruses in their phylum with enveloped virions, and they have molecular and biological characteristics likely inherited from an ancestor shared with the arthropod nege-like viruses. Kitavirids may be well-adapted to their mite vectors, whose fitness can be enhanced upon infection compared to their plant hosts, where they lack long-distance movement capability resulting in non-systemic infection (Ramos-González *et al*., 2023).

The present study reports the first detection of ToFBV in Switzerland. Reports of the virus from Italy and Brazil showed its recurrence on the infested sites (Ciuffo *et al.,* 2020; Kitajima *et al.,* 2022). The site where the Swiss isolate was identified will be monitored to increased knowledge of the epidemiology of the virus.

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New or Unusual Disease Reports

First report of Pythium root rot of hydroponic lettuce (*Lactuca sativa*) in Greece, caused by *Pythium* Cluster B2a sp.

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Summary. Pythium root rot has been reported in several countries, but in Greece this disease was first detected in 2021, causing severe yield losses in a hydroponic lettuce crop. Isolations, morphological and molecular characterization, as well as pathogenicity assays identified a *Pythium* Cluster B2a species causing the disease in hydroponically grown lettuce. This is the first report of *Pythium* Cluster B2a sp. causing lettuce root rot in Greece.

Keywords. Minor root pathogens, molecular characterization, pathogenicity assays.

INTRODUCTION

Hydroponic and soilless culture cropping systems occupy approx. 5% of the greenhouse area in Greece, with a tendency for further expansion, especially in new high-tech greenhouse facilities. The increasing interest in soilless production systems has led to development of specialized decision support systems, which provide full support in managing the nutrition of these crops through balanced nutrient solutions (Savvas *et al.*, 2023). Lettuce and other leafy vegetables are mainly produced in open fields and high tunnel houses in Greece, but there is an increasing tendency for year-round production in modern greenhouses using hydroponic technologies such as the Nutrient Film Technique (NFT) Barbosa *et al.*, 2015). The lack of contact with soil in hydroponic lettuce production considerably reduces infections by soil-borne pathogens, although hydroponics cannot fully eliminate this risk.

Pythium root rot has been a major concern for hydroponic lettuce growers, and has been reported to cause important yield losses in Italy, Cyprus, and Connecticut, United States of America, especially when warm temperatures occur (Garibaldi *et al.*, 2017; Pantelides *et al.*, 2017; McGehee *et al.*, 2018; Cacciola and Gullino, 2019). The disease has also been reported in hydroponi-

cally grown Welsh onions in Japan (Shimizu and Tojo, 2021). Currently, there are no approved fungicides for the control of the disease, but research has been conducted on effects of some biological control agents and chemicals to control the disease (Utkhede *et al.*, 2000).

MATERIALS AND METHODS

Sampling and isolation procedures

During September 2021, 30% of young 'Jokary' lettuce plants, grown hydroponically in an NFT system located in Viotia region, Greece, exhibited dark brown necrotic lesions scattered throughout the root system at approx. 2 weeks post transplantation (Figure 1A). In mature plants, the whole root systems were necrotic, with reduced biomass compared to apparently healthy plants (Figure 1B). In both cases, older leaves on the affected plants were chlorotic and wilted. Isolations were conducted onto V8-PAR medium, selective for oomycetes (Jeffers, 1986). For these, diseased roots were removed, washed with running tap water for 30 min and surface sterilized by immersion in 10% sodium hypochlorite for 2 min, 70% ethanol for 3 min, and then rinsing twice with sterile double distilled water. Small fragments of sterilized roots were placed onto V8-PAR medium and incubated at 25°C with a 12 h photoperiod.

DNA extraction and PCR amplification

Total genomic DNA was extracted from selected isolates according to Zelaya-Molina *et al*. (2011). An initial PCR assay was carried out using the universal primer set ITS5 and ITS4 (White *et al.*, 1990) targeting the internal transcribed spacer region 1 and 2 containing the 5.8S region (Table 1). PCR products were precipitated using ammonium acetate and subjected to sequencing. The derived consensus sequences were edited using Benchling software (https://www.benchling.com/), and were compared to GenBank database sequences by BLASTn analysis. Due to the incapability of ITS region sequencing for identifying the isolated oomycete to the species level, additional molecular markers were employed. Cytochrome b oxidase subunits 1 and 2 (COI1, COI2), which are accepted for oomycete barcoding, were amplified by PCR using appropriate primers (Table 1).

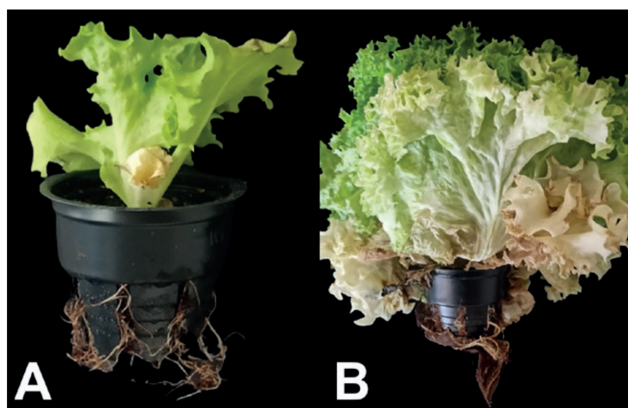

Figure 1. A) 2-week-old hydroponically grown lettuce plant with dark brown lesions in the roots and wilting of the lower leaves. B) mature plant showing chlorosis, yellowing, and wilting symptoms of the lower leaves, while the whole root system is necrotic.

PCR products were purified and sequenced as described above. Sequences of ITS and cytochrome b oxidase subunits 1 and 2 were deposited in GenBank under the respective accession numbers OQ657948, OQ686764 and OQ686765.

Phylogenetic analysis

Phylogenetic analyses were conducted using the MEGA-X Software (Kumar *et al*., 2018). Phylogenies were inferred using the p-distance substitution model for the three loci using the Neighbor-Joining (NJ) statistical method and 1,000 bootstrap replications. Analyses were also run using the concatenated sequences of ITS, COI1 and COI2, utilizing the p-distance substitution model to construct phylogenetic trees using the NJ statistical method with 1,000 bootstrap replications.

Pathogenicity assays

Pathogenicity tests were conducted on 'Jokary' lettuce plants to investigate the role of the isolated oomycete species in disease development. Eight 2-week-old lettuce plants were grown in 60 × 39 × 20 cm (l/w/h) tanks (one with eight inoculated plants, and the other with eight non-inoculated plants), each containing a standard nutrient solution for commercial crops (pH = 5.6, EC = 2.63 dS m^{-1}, K^+ 10.20 mmol L^{-1}, Ca^{2+} 4.86 mmol L^{-1}, Mg^{2+} 1.20 mmol L^{-1}, NH_4^+ 1.81 mmolL^{-1}, SO_4^{2-} 1.45 mmolL^{-1}, NO_3^- 16.78 mmolL^{-1}, $H_2PO_4^-$ 1.36 mmolL^{-1}, Fe 62.27 mmolL^{-1}, Mn^{2+} 5 mmolL^{-1}, Zn^{2+} 4 mmolL^{-1}, Cu^{2+} 0.71 mmolL^{-1}, B 43.8 mmolL^{-1}, Mo 0.70 mmolL^{-1}, Cl^- 2.75 mmolL^{-1}, Na^+ 0.20 mmolL^{-1} and HCO_3^- 1.03 mmolL^{-1}). For oxygen flow and nutrient circulation, two air pumps were used in each tank to provide enough oxygen for the plants. For pathogen inoculum, two intact cultures of a *Pythium* Cluster B2a sp. isolate from

Table 1. Primer sets used for phylogenetic analysis of oomycete species isolated from diseased lettuce plants.

Primer	Strand	Target	Sequence (5' -> 3')	Reference
ITS5	Forward	ITS	GGAGTAAAAGTCGTAACAAGG	White *et al.*, 1990
ITS4	Reverse		TCCTCCGCTTATTGATATGC	
OomCoxILevup	Forward	Cytochrome oxidase subunit 1	TCAWCWMGATGGCTTTTTTCAAC	Robideau *et al.*, 2011
OomCoxI-Levlo	Reverse		CYTCHGGRTGWCCRAAAAACCAAA	
Cox2F	Forward	Cytochrome oxidase subunit 2	GGCAAATGGGTTTTCAAGATCC	Choi *et al.*, 2015
Cox2RC4	Reverse		TGATTWAYNCCACAAATTTCRCTACATTG	

90 mm Petri dishes were mixed with 250 mL of nutrient solution and homogenized using a mixer. The mix containing the pathogen and nutrient solution was then poured into a tank (total volume of 3.5 L), while pure nutrient solution was added into a tank containing the non-inoculated control plants. The plants were then kept in a greenhouse at 25°C for 4 weeks.

RESULTS AND DISCUSSION

Forty-eight hours after isolation, oomycete-like colonies were observed on isolation plates, and these were transferred to new V8-PAR or PDA plates. The isolates produced white, flat, rosette-like mycelium on PDA (Figure 2), while on V8-PAR the mycelium was white and flat. Under microscopic observation, the colonies produced filamentous sporangia that formed dendroid structures and intercalary oogonia with straight oogonial stalks, of about 21 μm diameter. Antheridia were monoclinous and more than one antheridium per oogonium was observed. Oospores, while rarely developed, were plerotic or almost plerotic and uncoloured. Based on morphology, identification of the isolates to the species level was not possible, so molecular identification methods were used.

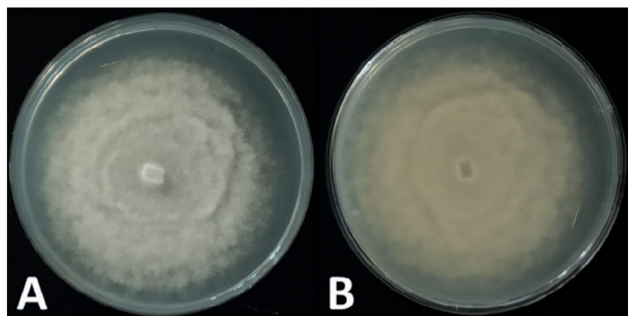

Figure 2. *Pythium* Cluster B2a sp. colony in PDA. A) front side and B) rear side of *Pythium* Cluster B2a sp. isolated from the roots of diseased lettuce plants.

BLASTn analysis against GenBank database sequences revealed 100% identity of the isolates obtained with the *Pythium* species *P. dissotocum*, *P. diclinum*, *P. coloratum and P. lutarium*. According to Robideau *et al.* (2011), the above species belong to a group indicated as *Pythium* Cluster B2a, and these organisms are indistinguishable based only on their ITS regions. Phylogenetic analysis carried out with the three genetic loci separately or in concatenation produced similar topologies (Figure 3A and 3B). Based on the evolutionary distances computed with the p-distance method for ITS locus and the concatenated sequences, the present study isolate belongs to the *Pythium* Cluster B2a species complex, and is closely related to *P. diclinum* with an evolutionary p-distance of 0 to $1{,}49 \times 10^{-3}$, respectively (Supplementary Table 1 and 2).

Approximately 10 days post-inoculation, plants inoculated with *Pythium* Cluster B2a sp. isolate showed chlorosis, yellowing and wilting symptoms of the lower leaves near the crowns, while their roots became brownish and eventually necrotic (Figure 4). Two weeks post-inoculation, almost all the inoculated plants collapsed, while control plants showed no symptoms. To fulfill Koch's postulates, isolations were carried out from the inoculated and control plants into corn meal agar (CMA) containing tetracycline hydrochloride 0.01% v/v). Isolations from the inoculated plants yielded pure cultures identical to *Pythium* Cluster B2a sp. while no microbial growth was observed from the isolations conducted from the control plants, thus fulfilling Koch's postulates.

In some cases, *Pythium* species are considered as "minor root pathogens" (Stanghellini, 1986), but favourable conditions for the pathogen may lead to significant yield losses. Although prevalence of Pythium root rots in hydroponically grown lettuce is limited in Greece, it is important that the pathogen biology and the conditions leading to disease outbreaks are understood.

This is the first report of *Pythium* sp. belonging to *Pythium* Cluster B2a causing root rot in hydroponically grown lettuce in Greece.

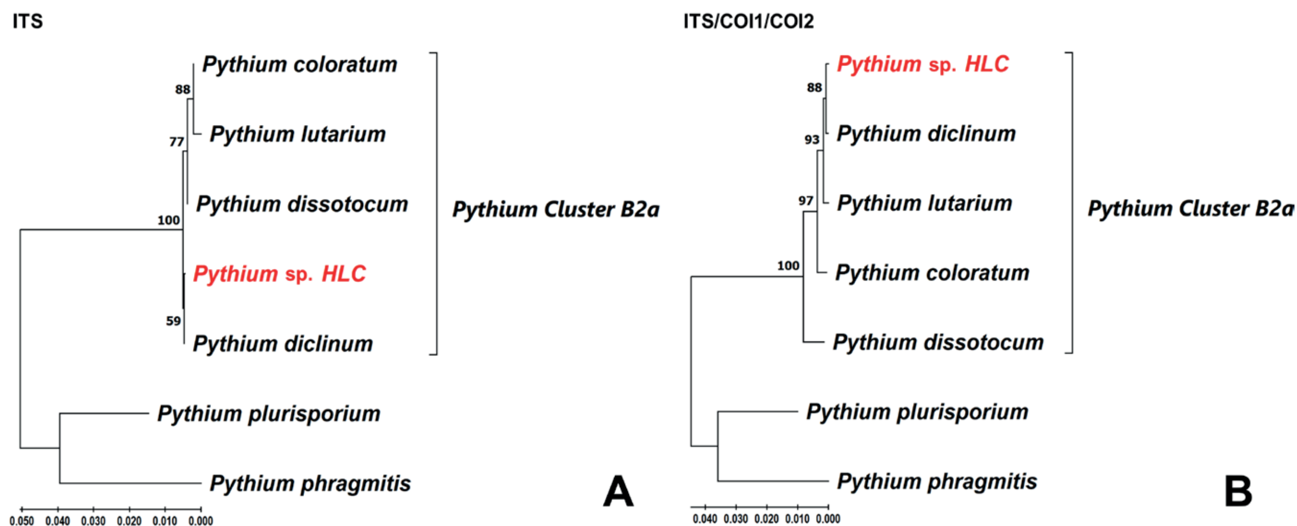

Figure 3. A) Phylogenetic tree of *Pythium* Cluster B2a sp. generated using the ITS sequences. B) phylogenetic tree of *Pythium* Cluster B2a sp. generated using the concatenated sequences. Phylogeny was inferred using the Neighbor-Joining method (NJ) with 1,000 bootstrap replications utilizing the p-distance substitution model. The optimal trees are shown. The phylogenetic analyses involved seven sequences with 894 final nucleotide positions for the ITS, and 2150 final nucleotide positions for the concatenated sequences. The OTU highlighted in red color represents the species isolated in this study. *Pythium plurisporum* and *Pythium phragmitis* were used as outgroups. The numbers on branches represent the bootstrap values.

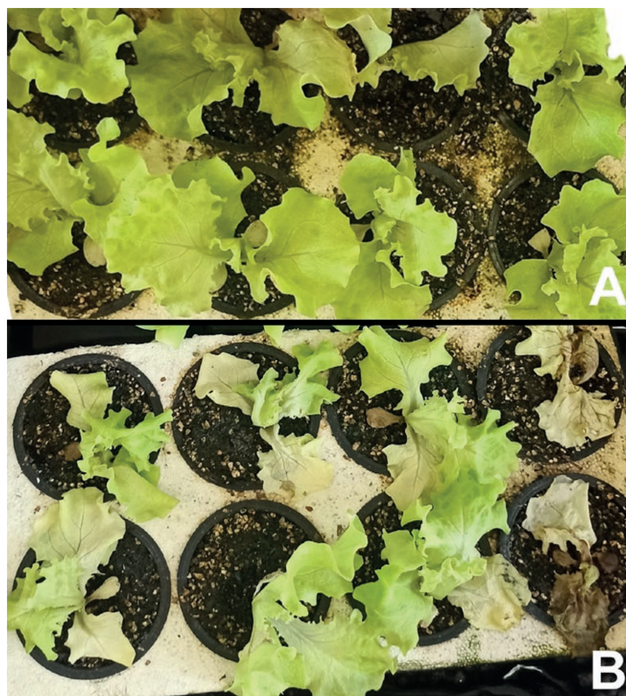

Figure 4. Pathogenicity assays with *Pythium* Cluster B2a sp. Images taken 2 weeks post inoculation with the pathogen. A) Control (non-inoculated) lettuce plants, and B) plants inoculated with *Pythium* Cluster B2a sp.

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Research Papers

Trichoderma in the Maltese Islands

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Summary. This study assessed presence of *Trichoderma* spp. in the Maltese Islands. Isolates were identified using dichotomous keys and DNA barcoding. Ten distinct isolates were obtained from different soils and other substrates, and were identified as *T. virens, T. citrinoviride, T. gamsii,* and, in the former *T. harzianum* species complex, *T. breve, T. afroharzianum* and *T. atrobrunneum*. Five out of these six fungi are reported for the first time in the Maltese Islands, and *T. brevis* is reported for the first time in Europe.

Keywords. ITS, *tef1*.

INTRODUCTION

The Maltese Islands are located in the central Mediterranean Sea, and together have a land area of 316 km^2 aligned in a NW-SE direction (Schembri, 1996). The climate of these islands is strongly bi-seasonal, with a hot, dry season from April to mid-September each year, and a mild wet season from mid-September to March. Relative humidity is high throughout the year, in the range of 65% to 80% (Galdies, 2011).

The known fungal diversity of the Maltese Islands includes approx. 400 macrofungal taxa, while the recorded list of microfungi species is incomplete and many remain unidentified or inadequately described. An extensive historical excursus for Maltese mycological studies was provided by Mifsud (2022), but only four studies have dealt with the microfungi on these islands (Saccardo, 1912, 1914, 1915; Porta-Puglia and Mifsud, 2006). Porta-Puglia and Mifsud (2006) reported for the first time the species *Trichoderma harzianum* Rifai (*Sordariomycetes*, *Hypocreales*, *Hypocreaceae*) as part of a checklist of microfungi of the Maltese Islands.

Trichoderma spp. are free-living, filamentous Ascomycetes with worldwide distributions. They grow rapidly, have bright green to white conidia and repeatedly branched conidiophores bearing phialides (Gams and Bissett, 1998). This genus was first described by Persoon (1794) and later by Rifai (1969). *Trichoderma* spp. can often occur on decaying wood and other sources of cellulose, including those occurring in soils (Kubicek *et al.*, 2008; Jaklitsch, 2009).

These fungi have also been isolated from unusual sources, including the guts of cellulose consuming insects such as cockroaches and termites, as well as marine mussels and sponges (Sallenave and Pouchus, 1999; Sallenave-Namont and Pouchus, 2000; Yoder *et al*., 2008; Guswenrivo *et al*., 2018; Yamada *et al*., 2019). More than 360 species have been described within *Trichoderma*, and several new species are recognized using molecular taxonomy (Bissett *et al*., 2015; Cai and Druzhinina, 2021). For *Trichoderma* taxonomy, the primary DNA barcoding loci for molecular identification are the complete sequences of the rRNA internal transcribed spacers 1 and 2 (ITS1 and ITS2), which also include the respective sequences of the genes encoding 5.8 S rRNA (Schoch *et al*., 2012). Partial fragments of the translation elongation factor 1 alpha (tef1) gene (Druzhinina and Kubicek, 2005), and the RNA polymerase B subunit II (rpb2) gene (Liu *et al*., 1999; Druzhinina *et al*., 2006; Atanasova *et al*., 2013) are generally used as secondary DNA barcodes. Phylogeny analyses within *Trichoderma* have led to separation of species into clades, that are groups of species which each include a common ancestor (Druzhinina *et al*., 2006; Samuels *et al*., 2012).

The cladistics system for *Trichoderma* has been revised, leading to the arrangement of all known *Trichoderma* species in different PhyloOrders based on the concept of genealogical concordance for phylogenetic species recognition (GCPSR) (Cai and Druzhinina, 2021). In the PhyloOrder system, species are ordered on a whole genus *rpb2* phylogram, and the PhyloOrder category determines neighbouring species. The taxonomy of *Trichoderma* currently accepted by the International Commission on *Trichoderma* Taxonomy (ICTT) assigns *Trichoderma* species to six PhyloOrders (https://trichokey.com/index.php/trichoderma-taxonomy-2020; last accessed 27 January, 2023).

The *Trichoderm*a species reported from the Maltese Islands are *T. harzianum* Rifai (*Sordariomycetes*, *Hypocreales*, *Hypocreaceae*) and *T. viride*, that were recorded by Porta-Puglia and Mifsud (2006). However, recent studies have discriminated several cryptic species based on molecular characterization, to the point where *Trichoderma* is referred to as a species complex, and its taxonomy is not considered as definitely set (Chaverri *et al*., 2003; Samuels, 2006; Druzhinina *et al*., 2010). Furthermore, it is probable that isolated areas, like islands, host *Trichoderma* strains or ecotypes with physiological and metabolic adaptations peculiar to the particular ecological and climatic features of each island.

The present study included a survey of occurrence of *Trichoderma* species in five soil samples and other organic substrates collected from distinct habitats in the Maltese Islands.

MATERIALS AND METHODS

Soil sampling for Trichoderma *spp.*

For isolation of *Trichoderma* spp., soil samples (each approx. 200 g) were collected from five locations in the Maltese Islands during the rainy season commencing from September 2017. The sampling locations selected were distinct habitats in the Maltese Islands, namely coastal garrigue (Ix-Xagħra l-Ħamra), the wet valley and ridge areas of a semi-natural woodland (Buskett garden), a man-made stand of *Pinus halepensis* (Floriana), and the Argotti Botanic Garden which hosts a large number of indigenous and exotic plant species. One gram sub-samples from each field soil sample were processed within 48 h from collection, and the remaining amount of each sample was preserved at 4°C in a pre-sterilized contain. Five other non-soil substrates were also sampled (Table 1 and Figure 1).

Table 1. Soil and other substrates assayed for *Trichoderma* isolates.

Sample	Origin	Sampling Location	Sample location coordinates
1	Soil	Ix-Xagħra l-Ħamra	35.95014°N; 14.34377°E
2	Soil	Floriana, Pinetum	35.89057°N; 14.50062°E
3	Soil	Buskett garden	35.85617°N; 14.39785°E
4	Soil	Buskett garden	35.85918°N; 14.39738°E
5	Coffee grounds	Argotti Botanic Garden	35.89239°N; 14.50300°E
6	*Aurificaria euphoriae* (Pat.) Ryvarden, basidiome	Argotti Botanic Garden	35.89239°N; 14.50300°E
7	Imported commercial compost (MXS Mikskaar, Tallinn, Estonia)	Argotti Botanic Garden	35.89239°N; 14.50300°E
8	*Euphorbia abyssinica* J.F. Gmel. trunk	Argotti Botanic Garden	35.89239°N; 14.50300°E
9	*Salsola melitensis* Botsch., trunk	Argotti Botanic Garden	35.89239°N; 14.50300°E
10	*Anacamptis pyramidalis* (L.) Rich., roots	Wied Babu	35.82191°N; 14.46021°E

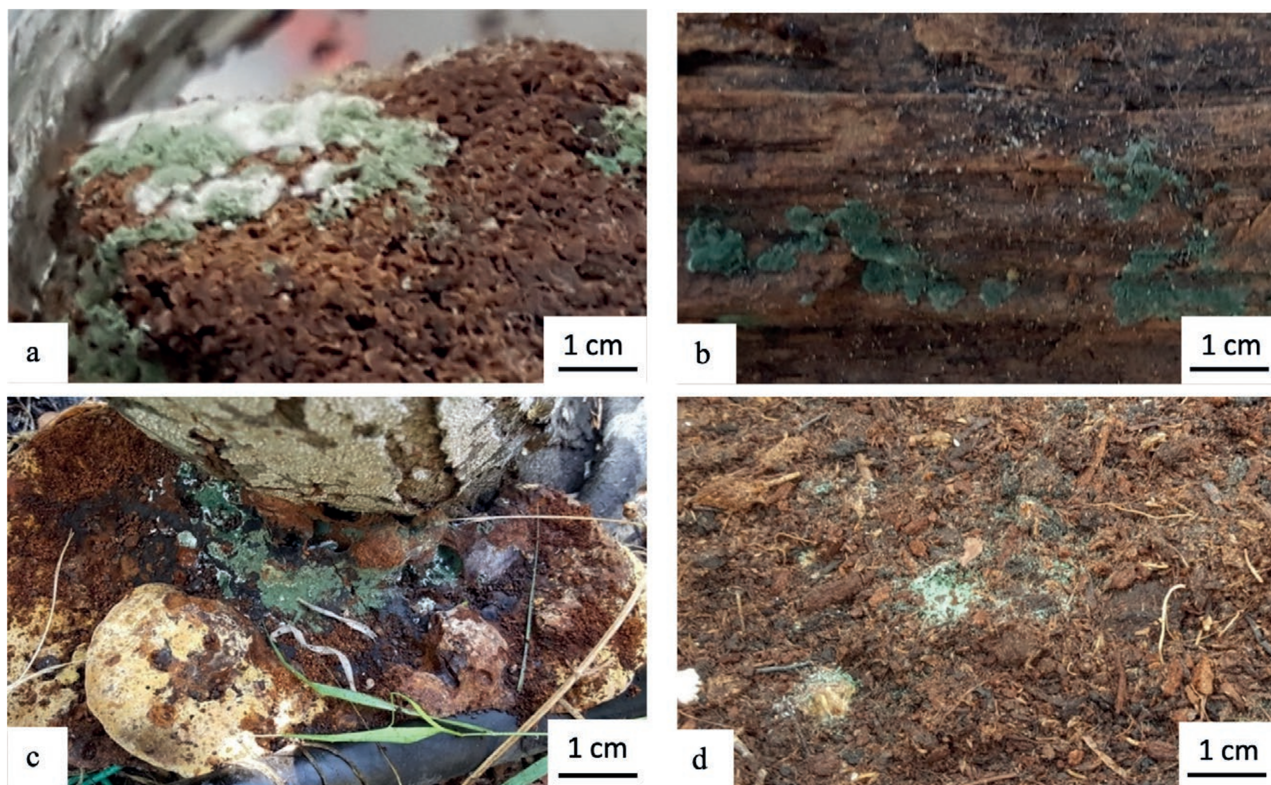

Figure 1. *Trichoderma* spp. growing on different substrates: (a) coffee grounds; (b) *E. abyssinica* trunk; (c) basidiome of *A. euphoriae*; (d) imported commercial compost.

Trichoderma *monoconidial isolations and isolate preservation*

For *Trichoderma* monoconidial isolations, 1 g of soil was dried at 60°C for 24 h, and then mixed with 500 mL of sterile water and allowed to rest for 24 h. Four 1:10 serial dilutions in sterile distilled water were then prepared, and 100 µL of each dilution was then spread onto a Petri dish containing modified Trichoderma selective medium prepared according to the recipe of Smith *et al.* (1990), except for the fungicides used, which were 2.5 mL L^{-1} Teldor (Fenhexamid 50% w/w; Bayer) and 2.5 mL L^{-1} Previcur (Propamocarb 60% w/w; Bayer). The Petri dishes were then incubated at 25°C for 24 to 48 h in the light, and were checked daily for colony growth. Single colonies were each transferred to a separate Petri dish containing potato dextrose agar, which had been prepared according to the manufacturer's instructions and supplemented with 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Genesee Scientific). The isolation plates were then incubated at 25°C in light.

From each antibiotic medium culture, a small piece (2 mm^2) of mycelium was aseptically transferred to a labelled sterile tube containing 10 mL of sterile distilled water. The tube was vortexed for 20 sec and then serially diluted to 10^{-1} and 10^{-2}. Aliquots (100 µL) were evenly spread on 2% Water Agar in Petri dishes, which were placed in an incubator (MLR 352 PHCBI, Tokyo, Japan) at 25°C and 70% RH under 800 lux fluorescent lamps. After 24 h, the dishes were aseptically examined under a stereomicroscope and checked for individual germinated conidia that were separated from each other. A small piece of agar bearing a single germinated conidium was then excised with a sterile lancet and transferred onto Potato Dextrose Agar with antibiotics (as above), and incubated at 25°C. Colony growth was followed for 21 d, and the colony growth pattern, conidium colour, conidiation pattern, and reverse colour were recorded for each isolate. The micromorphological features of each isolate were also observed under a microscope, using fragments of colonies collected axenically from the conidiation area contour, and were suspended in distilled water. Monoconidial cultures of the isolated fungi were stored at 4°C in test tubes containing Synthetic Nutrient Agar prepared according to Elad *et al.* (1981). Long-term preservation of *Trichoderma* isolates was carried out in sterile 99% glycerol stored at -18°C, according to Stocco *et al.* (2010). Voucher specimens

and isolates are conserved in the collection of Maltese mycoflora, hosted at the Seed Bank of the Department of Biology of the University of Malta, Valletta, Malta, under the accession codes listed in Table 2.

Molecular identification of Trichoderma *isolates*

Molecular identification at species level of the *Trichoderma* isolates was carried out using gene sequencing. Single conidium cultures grown on PDA at 25°C for 6 d were preserved in ethanol, and the ethanol fixed tissues was aseptically dissected into small sections using a sterile scalpel. All samples were processed for DNA extraction using the NucleoSpin Plant Kit (Macherey-Nagel) according to manufacturer instructions.

The ribosomal region including internal transcribed spacers ITS1 and ITS2, and the small subunit ribosomal RNA 5.8S (ITS) were amplified by PCR using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), ITS2 (5'- GCTGCGTTCTTCTTCATCGATGC-3'), and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). All amplifications were each carried out using the AccuStartTMII PCR ToughMix (Quantabio), in a final volume of 25 μL, containing 1 μL of each primer (10 pmol μL^{-1}) and 1 to 2 μL of DNA template. The PCR conditions were set to an initial denaturation temperature of 94°C for 5 min followed by 35 cycles each of 30 s at 94°C, 40 s at 48°C and 50 s at 72°C, with a final elongation phase of 7 min at 72°C. PCR products were visualized using electrophoresis on 1.5% agarose gels. For each successful PCR, 10 μL of PCR product were purified with a 2.5 μL mix containing exonuclease I (20 U μL^{-1}) and alkaline phosphatase (1 U μL^{-1}), using an incubation of 15 min at 37°C and 20 min at 75°C.

A fragment of the protein-coding translational elongation factor 1 alpha gene (tef1) was amplified by PCR using the primers EF1-1018F (5'-GAYTTCATCAAGAACATGAT-3') and EF1-1620R (5'-GACGTTGAADCCRACRTTGTC-3') (Stielow *et al.*, 2015). All amplifications were each carried out using the AccuStartTMII PCR ToughMix (Quantabioin a final volume of 25 μL, containing 1 μL of each primer (10 pmol μL^{-1}) and 1 to 2 μL of DNA template. The PCRs were set to initial denaturation at 94 °C for 5 min followed by 35 cycles each of 30 s at 94°C, 40 s at 48°C and 50 s at 72°C, with a final elongation phase of 7 min at 72°C. PCR products were visualized using electrophoresis on 1.5% agarose gels. For each successful PCR, 10 μL of PCR product were purified with a 2.5 μL mix containing exonuclease I (20 U μL^{-1}) and alkaline phosphatase (1 U μL^{-1}) using incubation of 15 min at 37°C and then 20 min at 75°C. All purified PCR products were sequenced in both forward and reverse directions by Macrogen Inc. (Amsterdam, the Netherlands), using M13 universal primers.

Forward and reverse sequences were assembled using Geneious (v. R10, Biomatters), and were reciprocally verified to generate a complete contig of each sequenced fragment. All contigs were then exported in FASTA format and compared with the GenBank reference database for taxonomic assignment using the BLAST algorithmus (Altschul *et al.*, 1990). The TrichOKey (http://isth.info/tools/molkey/index.php) and TrichoBLAST (http://www.isth.info/tools/blast/) tools were used to compare the ITS and tef1 sequences for species identification. In addition, a dataset of combined ITS and tef1 sequences was generated for eight *Trichoderma* isolates from the Maltese Islands. Furthermore, ten *Trichoderma* species reference strains, including *T. atrobrunneum* T57, *T. harzianum* CBS 226.95, *T. harzianum* HZA11, *T. afroharzianum* TB1-26, *T. breve* HMAS 248844, *T. zelobreve* CGMCC 3.19696, *T. virens Gv29-8*, *T. citrinoviride* HZA9, *T. neokoningii* CBS 120070, and *T. gamsii* GJS 05-111, were included in the analysis. *Cladobotryum heterosporum* CBS 719-88 was used as the outgroup. The multiple alignment of the combined sequence dataset (total 1226 nucleotide sites), performed with MUSCLE algorithm, and phylogenetic analysis using the Maximum Likelihood method were both carried out using MEGA11 software (Tamura *et al.*, 2021). The accuracy of the analyses were assessed using the bootstrap method with 1000 replicates.

A small fungal collection was established at the Department of Biology, University of Malta, where cultures of *T. atrobrunneum*, *T. afroharzianum, T. gamsii, T. breve, T. citrinoviride, T. virens* and *T. gamsii* are maintained as a living collection on different substrates and as samples held at different storage temperatures.

RESULTS

Ten *Trichoderma* strains were isolated from different locations and substrates in the Maltese Islands. Four of the strains were isolated from soils from different sampling locations. Four strains were isolated from a basidiome of *Aurificaria euphoriae*, from wood of two different dead trees, and from a commercial potting compost, all originating from Argotti Botanic Garden. One strain was isolated from coffee grounds, and one was isolated from *A. pyramidalis* roots (Table 1 and Figure 1). Each sample yielded a single isolate. The growth patterns and colours of top and reverse sides of Petri dish cultures of the *Trichoderma* spp. isolates grown on PDA and recorded at 3 d intervals up to 11 d are shown in Figure 2.

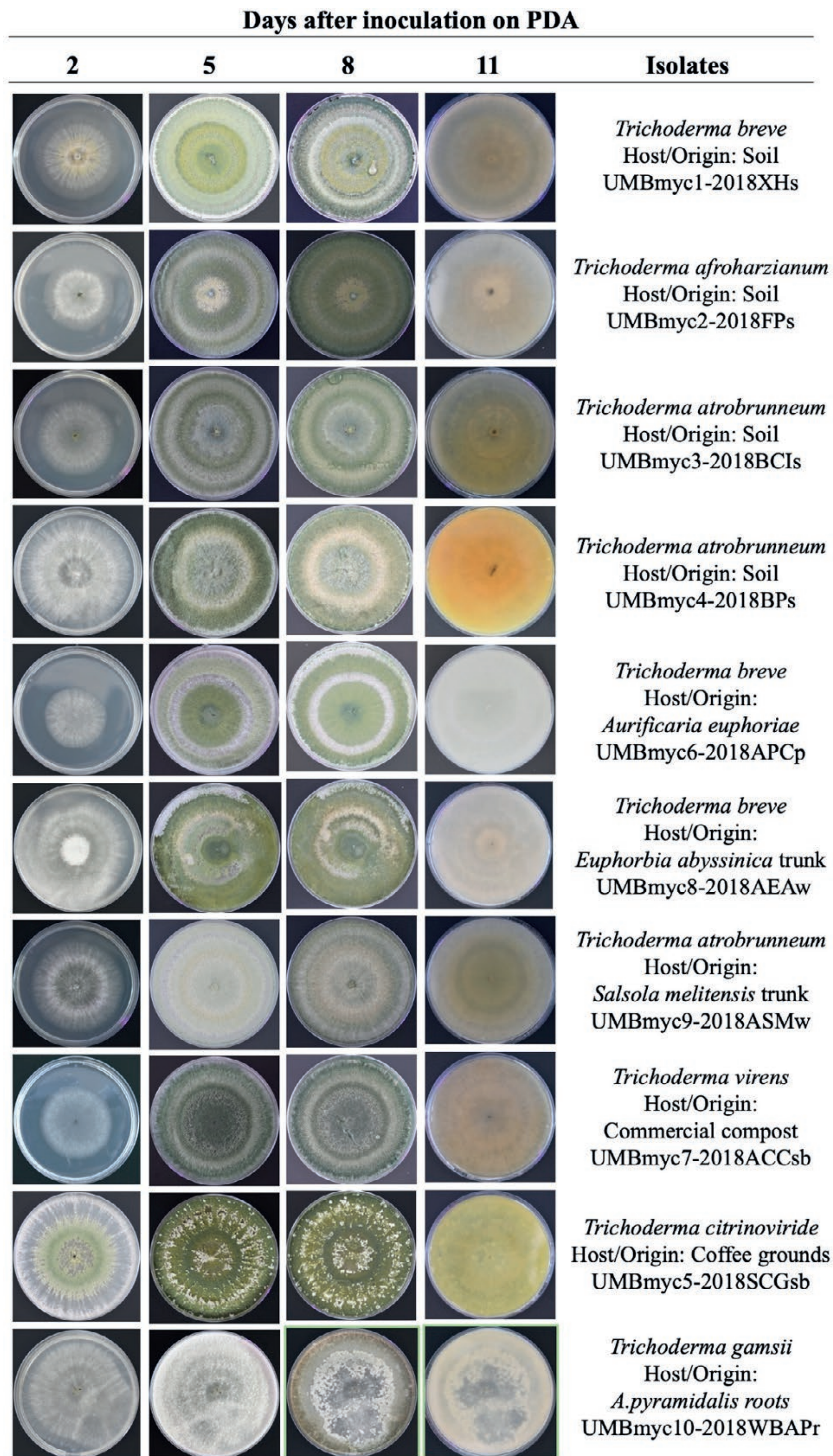

Figure 2. Top views of Petri plate cultures *Trichoderma* isolates grown on PDA for 2, 5, and 8 d. Colony reverse sides at day 11 are also shown, except for the *T. gamsii* colony, shown at day 21.

The isolates were identified using DNA sequencing. Sequences of ITS regions were used for preliminary identification at species level, based on BLAST analyses which only allowed definite identification of two isolates, UMBmyc5-2018SCGsb as *T. citrinoviride* Bissett, and UMBmyc7-2018ACCsb as *T. viride* Pers., with the remaining isolates identifying as *T. harzianum*.

Sequencing of tef1 gene was necessary to further differentiate within the *T. harzianum* species complex. Phylogenetic analysis of the combined ITS and tef1 sequences, compared with available sequences of *Trichoderma* species used as references, allowed identification of all the *T. harzianum* complex isolates as cryptic species, namely *T. breve* K. Chen & W.Y. Zhuang, *T. afroharzianum* P. Chaverri, F.B. Rocha & I. Druzhinina, and *T. atrobrunneum* F.B. Rocha, P. Chaverri & W. Jaklitsch. As shown in the phylogenetic tree in Figure 3, three isolates (UMBmyc3-2018BCIs, UMBmyc4-2018BPs and UMBmyc9-2018ASMw) were identified as *T. atrobrunneum*; isolate UMBmyc2-2018FPs clustered with the *T. afroharzianum* reference strain, and the isolates UMBmyc1-2018XHs, UMBmyc6-2018APCp and UMBmyc8-2018AEAw grouped with *T. breve* and *T. zelobreve*. The isolate UMBmyc10-2018WBAPr, from *Anacamptis pyramidalis* roots, was identified as *T. gamsii* Samuels & Druzhinina.

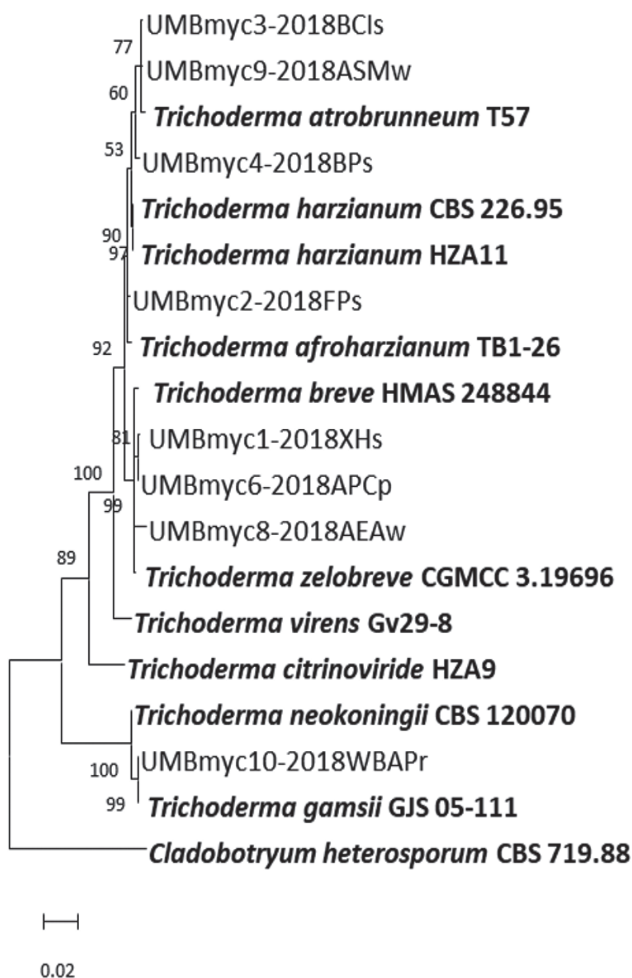

Figure 3. Phylogenetic tree for eight *Trichoderma* isolates, based on the combined sequences of ITS and tef1 gene fragments. The tree was obtained by using the Maximum Likelihood method and Tamura-Nei model. The proportions (%) in which the associated taxa clustered together are shown next to the branches, expressed as bootstrap values with 1000 replicates.

The assignment of species was carried out according to the current nomenclature defined by the International Commission on *Trichoderma* Taxonomy (ICTT; https://trichokey.com/index.php/trichoderma-taxonomy-2020, last accessed on 27 January, 2023). Among the six species identified in the Maltese Islands, listed in Table 2, *T. afroharzianum*, *T. atrobrunneum*, *T. breve* and *T. virens* belong to PhyloOrder clade 1, based on phylogeny of the currently rpb2-barcoded *Trichoderma* species. *Trichoderma citrinoviride* was assigned to PhyloOrder clade 3, and *T. gamsii* was assigned to PhyloOrder clade 5.

Nucleotide sequences were submitted to the GenBank Database with accession numbers from OQ378924 to OQ378933 for ITS (ten sequences) and from OQ384109 to OQ384116 for tef1 (eight sequences).

DISCUSSION

The Convention of Biological Diversity states that "Islands and their surrounding near-shore marine areas constitute unique ecosystems often comprising many plant and animal species that are endemic, and therefore found nowhere else on Earth" (Convention of Biological Diversity, https://www.cbd.int/island/). For these reasons, survey, cataloguing and preservation of biodiversity is important for small islands like the Maltase Islands. A multilocus identification system for *Trichoderma* (MIST), based on three phylogenetic marker databases (ITS, tef, and rpb2), is regarded as a valid tool for identification of *Trichoderma* species (Hatvani *et al.*, 2014). The genealogical concordance for phylogenetic species recognition (GCPSR) (Cai and Druzhinina, 2021) is the most widely accepted approach for *Trichoderma* identification, mostly to detect cryptic species. Standardization of species recognition criteria and agreement between *Trichoderma* taxonomists allows unambiguous diagnoses of species (Cai and Druzhinina, 2021). According to ICTT nomenclature, the recognized species belonging to Harzianum and Virens Clades are joined in the same

Table 2. Species identification of the *Trichoderma* spp. isolates from the Maltase Islands, based on DNA barcoding, according to the International Commission on Trichoderma Taxonomy (ICTT).

Isolate No.[a]	Origin and sampling location	Species	PhyloOrder (ICTT)	GenBank sequence accession numbers	
				ITS	tef1
UMBmyc1-2018XHs	Soil, Ix- Xagħra l-Ħamra	*T. breve*	1	OQ378924	OQ384109
UMBmyc2-2018FPs	Soil Floriana pinetum	*T. afroharzianum*	1	OQ378925	OQ384110
UMBmyc3-2018BCIs	Soil, Buskett Garden	*T. atrobrunneum*	1	OQ378926	OQ384111
UMBmyc4-2018BPs	Soil, Buskett Garden	*T. atrobrunneum*	1	OQ378927	OQ384112
UMBmyc5-2018SCGsb	Spent coffee grounds	*T. citrinoviride*	3	OQ378928	-
UMBmyc6-2018APCp	*Aurificaria euphoriae* (Pat.) Ryvarden, ABG[b]	*T. breve*	1	OQ378929	OQ384113
UMBmyc7-2018ACCsb	Commercial compost, ABG	*T. virens*	1	OQ378930	-
UMBmyc8-2018AEAw	*Euphorbia abyssinica* J.F. Gmel., ABG	*T. breve*	1	OQ378931	OQ384114
UMBmyc9-2018ASMw	*Salsola melitensis* Botsch., ABG	*T. atrobrunneum*	1	OQ378932	OQ384115
UMBmyc10-2018WBAPr	*Anacampis pyramydalis* (L.), Wied Babu	*T. gamsii*	5	OQ378933	OQ384116

[a] Accession No. in the collection of Maltese mycoflora, Seed Bank of the Department of Biology, University of Malta, Valletta, Malta.
[b] ABG = Argotti Botanical Garden.

PhyloOrder clade, named 1 (Cai and Druzhinina, 2021). Two species (*T. citrinoviride* and *T. gamsii*) belonging, respectively, to PhyloOrder clades 3 and 5, were identified among the Maltese isolates.

The present study used the ITS and tef1 sequences, and subsequently the *ITS4* and *TEF1α* sequences, to define the biodiversity of *Trichoderma* in the Maltese Islands. These phylogenetic analyses allowed identification of Maltese isolates at species level. Although only ten isolates were studied, they were identified as four different phylogenetic Clades. Seven out of the belonged to the Harzianum Clade, which so far is the most common and widespread. Three other isolates were assigned to the Virens, Longibrachiatum and Viride Clades. Based on currently accepted nomenclature and taxonomy, the Maltese isolates belonged to six different PhyloOrders (Cai and Druzhinina, 2021). In particular, the isolate from coffee was identified as *T. citrinoviride* (Longibrachiatum Clade), a very common soil fungus and also detected as an opportunistic pathogen of immunocompromised humans (Hatvani *et al.*, 2019). The isolate from compost was identified as *T. virens* (Virens Clade), a species commonly used as a biocontrol agent to protect various crops from a number of plant pathogens, and which has been utilized as a model for elucidating the mechanisms of biological control (Druzhinina *et al.*, 2011). The endophytic isolate from orchid roots was identified as *T. gamsii* (Viride Clade). The Viride Clade is the largest and the most diverse group of *Trichoderma*, characterized by species producing a wide range of bioactive compounds (Marik *et al.*, 2018).

Seven of the ten isolates, initially identified using ITS regions, belonged to the *T. harzianum* species complex, while the other three were *T. virens*, *T. gamsii* or *T. citrinoviride*. The seven isolates thus belonging to the *T. harzianum* species complex showed considerable phenotypic variation (Figure 2), which is consistent with findings of other authors (Chaverri and Samuels, 2003; Evans *et al.*, 2003; Samuels, 2006; Hoyos-Carvajal *et al.*, 2009; Jaklitsch, 2009; Gazis and Chaverri, 2010; Druzhinina *et al.*, 2011). The subsequent molecular analyses including tef1 sequencing, allowed differentiation of the isolates into three cryptic species, namely *T. afroharzianum, T. atrobrunneum* or *T. breve*. These results confirm the importance of tef1 sequences for studies of phylogeny and taxonomic characterization in *Trichoderma*.

While all the species isolated in the Maltese Islands are ubiquitous and have been reported from many world regions, *T. breve* was previously reported only from China, where it was first described in 2017 (Chen and Zhuang, 2017), and from central Africa where it was recovered as an endophyte of *Coffea* (del Carmen H. Rodríguez *et al.*, 2021). Thus, *T. breve* is reported here for the first time in Europe, and this report increases the list of *Trichoderma* species that occur in the European geographical areas (Jaklitsch, 2009, 2011; Jaklitsch and Voglmayr, 2015). Although similar to the *T. harzianum* species complex for morphology and culture traits, *T. breve* is phylogenetically more closely related to *T. bannaense*, another newly described species from China, than to *T. harzianum* (Chen and Zhuang, 2017). The Maltese isolates of *T. breve* were from soil, from a dead branch of *E. abyssinica*, and from the polypore fungus *A. euphoriae* growing on *Prunus cerasifera*, suggesting that *T. breve* may exhibit more than one ecological habit.

All of the other *Trichoderma* species isolated in the present study, namely *T. afroharzianum*, *T. atrobrunneum*, *T. citrinoviride*, *T. virens*, and *T. gamsii*, have been extensively described and isolated from a number of geographical areas and substrates (Chaverri and Samuels, 2003; Jaklitsch *et al.*, 2006; Chaverri *et al.*, 2015). Due to the peculiar environmental and climatic features of the Maltese Islands, these isolates may have beneficial properties and also resilience to abiotic stresses that occur in the Mediterranean basin, such as drought, heat stress and salinity, making them suitable for applications where climate change and global warming prescribe potential biotechnology applications.

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Research Papers

Cercospora beticola causes leaf and stem spots of New Zealand spinach (*Tetragonia tetragonoides*) in Brazil

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Summary. New Zealand (NZ) spinach (*Tetragonia tetragonoides*) is an important leafy vegetable crop in Brazil and other countries. This plant is used as a substitute for common spinach because it is rustic and tolerant to tropical and subtropical environmental conditions. It is often affected by a leaf and stem spot disease, which increases in severity during the warm climatic periods. *Cercospora tetragoniae* has been reported as the cause of this disease, but this is based on an early description of a *Cercospora*-like species on this host in Argentina, first named *Cercosporina tetragoniae* but later recombined into *Cercospora*. In the present study, isolates of *Cercospora*-like fungi were obtained from NZ spinach and beetroot plants in Brazil, and a multigene molecular study including the *act, cal, gapdh, his3,* ITS, and *tef1-α* regions was carried out to identify the causative pathogen. Additionally, morphological and cross inoculation studies were conducted with isolates obtained from diseased plants. The pathogen was confirmed as *Cercospora beticola*, a common and harmful pathogen of beetroot (*Beta vulgaris*). Cross-inoculations of isolates obtained from NZ spinach and beetroot showed that the isolates are infective to both hosts. This increases knowledge of epidemiology and management of this important disease. Several attempts to re-collect samples from the type locality in Argentina failed. NZ spinach is no longer grown at La Plata (Argentina), the type locality of *C. tetragoniae*. Therefore, the task of re-collecting the pathogen is still pending, for epitype designation and for a full clarification of the taxonomic status of *C. tetragoniae.* The possibility of the pathogen being seed-transmitted has been assessed, and evidence obtained justifies further assessment of this aspect.

Keywords. *Aizoaceae*, *Amaranthaceae*, Cercosporoid fungi, *Beta vulgaris*.

INTRODUCTION

New Zealand (NZ) spinach (*Tetragonia tetragonoides, Aizoaceae*) is widely cultivated as a leafy vegetable. It is a semi-herbaceous, branched, succulent plant with a creeping growth habit and fleshy, triangular-shaped dark green leaves (Filgueira, 2000). It has a broad natural distribution ranging from sandy shorelines of eastern Asia to Australia and New Zealand, despite its

common name suggesting it to have a New Zealand origin (CABI, 2018). NZ spinach has been introduced and has become invasive in coastal habitats of Chile, Hawaii, Florida and California (CABI, 2018). The predominant "spinach" cultivated in Brazil is NZ spinach, and the area under cultivation was estimated to be approx. 1700 hectares, making it the fifth most important leafy vegetable in Brazil (Vilela and Luengo, 2017).

Little has been published on plant pathogens attacking NZ spinach. Only the leaf-spot fungus *Cercospora tetragoniae* (*Mycosphaerellaceae*) has been reported in association with this plant in Brazil (Viégas, 1945; Hino and Tokeshi, 1978; Mendes and Urben, 2019). In the first Brazilian report, Viégas (1945) provided a detailed description and illustration of the fungus on *T. tetragonoides* (as *T. exapansa*), based on samples collected in Campinas and São Paulo (state of São Paulo, Brazil). However, no pure cultures from Viégas's specimens or based on Hino and Tokeshi's records have been deposited in public culture collections (Viégas, 1945; Hino and Tokeshi, 1978).

In August 2017, NZ spinach plants growing in the Infectarium, a disease-demonstration garden on the Universidade Federal de Viçosa campus (Viçosa, Minas Gerais, Brazil) were observed with leaf and stem spot symptoms. These symptoms increased in incidence and severity as the host plants aged and as temperatures and humidity increased (Figure 1, A and B). The diseases started as few spots on few isolated leaves, and these became progressively more abundant on leaves and also occurred on tender stems. At final stages, most stems were girdled by coalescing necroses, and stem dieback led to death of aerials part of most affected plants. Slow recovery, from remaining root systems and seeds from the previous season, was observed in cool months in the same plots. This disease progression was repeated in following years, both in the Infectarium and in commercial vegetable gardens of Viçosa and in Rio de Janeiro (municipalities of Petrópolis, Nova Friburgo, and elsewhere).

This disease commonly occurs in these areas and wherever NZ spinach is cultivated in Brazil, and is the most damaging disease attacking these vegetable crops in several Brazilian states (R. W. Barreto, personal observations). Specimens of diseased NZ spinach were collected for preliminary examination, and a dematiaceous hyphomycete was regularly found associated with the necrotic host tissues. The present paper outlines results of an investigation that aimed to provide clarification of the aetiology of this disease observed in Brazil.

MATERIALS AND METHODS

Isolation and morphological characterization of the pathogen

A sample of NZ spinach with leaf and stem spot symptoms at various stages of development was collected for laboratory examination, and selected parts with disease symptoms were dried in a plant press. Later, samples of NZ spinach bearing identical symptoms were obtained from a vegetable growing area in the separate geographic region of Petrópolis (state of Rio de Janeiro). A representative herbarium specimen from each source was deposited in the local herbarium of the Universidade Federal de Viçosa (Acc. Nos. VIC 44406, VIC 44456, VIC 44457, VIC 44458). A cercosporoid fungus was directly isolated from sporulating areas of lesions, by transfer of individual conidia onto potato dextrose agar (PDA; Kasvi) in Petri plates, using a sterile fine pointed needle, and pure cultures were obtained. Representative isolates of the fungus collected from NZ spinach at Viçosa and Petrópolis were deposited in the local culture collection (Acc. Nos. COAD 2380, COAD 2477), as well as pure cultures of *Cercospora beticola* obtained from diseased beetroot plants (*Beta vulgaris*) from the same localities (Acc. Nos. COAD2476, COAD2478).

In addition, fungal structures were scraped from the surfaces of the diseased NZ spinach tissues with a scalpel, and were mounted in lactoglycerol for microscope observations. Biometric data was compiled from at least 30 measurements of conidiophores and conidia. The samples were examined and images were captured using a light microscope (Olympus model BX 51) equipped with a digital image capture system (Olympus Q-Color 3™ camera). Morphology of colonies and colony pigmentation were observed after 7 d growth on PDA at 25°C under two fluorescent white and one NUV black light lamps (for 12 h each day), located 35 cm above the culture plates. Colony colour (Rayner, 1970) was assessed.

Detection of pathogen in seeds

A "blotter test" was carried out to preliminarily verify the speculation in Japan, in Table 1 of Hino and Tokeshi (1978), and in the present study in Brazil, that *Cercospora* spp. (including *C. tetragoniae*) occurring on plants in both countries may have been introduced at the same time through the transfer of "plant tissues or seeds". Packets of NZ spinach 'seed' (NZ spinach fruits which are used and treated as seeds for this crop), sold under three Brazilian brand names (Feltrin, Isla, Topseed) were acquired. Additionally, seeds obtained from plots at the Infectarium where NZ spinach showed disease symptoms

Table 1. Isolates included in the phylogenetic study reported in this paper. The newly generated sequences are underlined, and ex-type strains included in this study are indicated in bold font.

Fungal species	Strain number	Host name	GenBank Acc. No.					
			ACT	CAL	GAPDH	HIS3	ITS	tef1
Cercospora apii	**CBS 116455**	*Beta vulgaris*	AY840450	AY840417	MH496173	AY840384	AY840519	AY840486
	CCTU 1086	*Cynanchum acutum*	KJ885928	KJ885767	MH496176	KJ886089	KJ886411	KJ886250
	CCTU 1215	*Cynanchum acutum*	KJ885929	KJ885768	MH496177	KJ886090	KJ886412	KJ886251
Cercospora apiicola	**CBS 116457**	*Apium* sp.	AY840467	AY840434	-	AY840401	NR119526	AY840503
	CPC 11642	*Apium* sp.	DQ233393	DQ233419	-	DQ233441	DQ233341	DQ233367
Cercospora armoraceae	**CBS 250.67**	*Armoracia rusticana*	JX143053	JX142807	MH496181	JX142561	JX143545	JX143299
	CBS 555.71	*Coronilla varia*	JX143058	JX142812	MK531772	JX142566	JX143550	JX143304
Cercospora asparagi	AS16-01	*Asparagus officinalis*	KY549091	KY549093	-	KY549095	KY549097	KY549101
	AS16-02	*Asparagus officinalis*	KY549092	KY549094	-	KY549096	KY549098	KY549102
Cercospora beticola	**CBS 116456**	*Beta vulgaris*	AY840458	AY840425	MH496185	AY840392	NR_121315	AY840494
	CCTU 1088	*Sonchus asper*	KJ885945	KJ885784	MH496191	KJ886106	KJ886428	KJ886267
	CCTU 1089	*Plantago lanceolata*	KJ885946	KJ885785	MH496189	KJ886107	KJ886429	KJ886268
	COAD 2380	*Tetragonia tetragonoides*	OQ944120	MH469231	OQ944127	OQ944129	MG780415	MN517124
	COAD 2476	*Beta vulgaris*	OQ944121	MT561868	OQ944124	OQ944130	MT555312	MN517125
	COAD 2477	*Tetragonia tetragonoides*	OQ944122	MT561866	OQ944125	OQ944131	MT555313	-
	COAD 2478	*Beta vulgaris*	OQ944123	MT561867	OQ944126	OQ944128	MT555314	MT561869
Cercospora celosiae	CBS 132600	*Celosia argentea* var. *Cristata*	JX143080	JX142834	-	JX142588	JX143570	JX143326
Cercospora coniogrammes	**CBS 132634**	*Coniogramme japonica* var. *Gracilis*	JX143095	JX142849	-	JX142603	NR_147260	JX143341
	CPC 25070	*Hypolepis mitis*	KT037599	KT037466	-	-	KT037517	KT037477
Cercospora cf. *citrulina*	CBS 119395	*Musa* sp.	JX143089	JX142843	-	JX142597	EU514222	JX143335
	CBS 132669	*Musa* sp.	JX143090	JX142844	-	JX142598	-	JX143336
Cercospora gamsiana	CCTU 1074	*Malva neglecta*	KJ885943	KJ885782	MH496276	KJ886104	KJ886426	KJ886265
	CCTU 1035	*Malva sylvestris*	KJ885940	KJ885779	MH496277	KJ886101	KJ886423	KJ886262
	CCTU 1109	*Malva sylvestris*	KJ885948	KJ885787	MH496278	KJ886109	KJ886431	KJ886270
Cercospora cf. *malloti*	MUCC 575	*Cucumis melo*	JX143138	JX142892	-	JX142646	JX143625	JX143384
	MUCC 787	*Mallotus japonicus*	JX143139	JX142893	-	JX142647	JX143626	JX143385
Cercospora mercurialis	**CBS 550.71**	*Mercurialis perennis*	JX143141	JX142895	-	JX142649	JX143628	JX143387
	CBS 551.71	*Mercurialis ovata*	JX143142	JX142896	-	JX142650	JX143629	JX143388
	IRAN 3949C	*Mercurialis annua*	MT843620	MT843648	MT843715	MT843673	MT804381	MT843593
Cercospora cf. *richardiicola*	CCTU 1004	*Bidens tripartita*	KJ886036	KJ885875	MH496295	KJ886197	KJ886519	KJ886358
	CBS 132627	*Ajuga multiflora*	JX143153	JX142907	-	JX142661	JX143640	JX143399
Cercospora samambaiae	**CPC 24673**	*Thelypteris dentata*	KT037596	KT037463	-	KT037555	KT037514	KT037474
	COAD 1427	*Pteris deflexa*	KT037590	KT037457	-	-	KT037508	KT037468
Cercospora cf. *sigesbeckiae*	CBS 132641	*Persicaria orientalis*	JX143166	JX142920	-	JX142674	JX143653	JX143412
	IRAN 3832C	*Glycine max*	MT186115	MT186086	MT186131	MT186076	MT338034	MT186099
	IRAN 3837C	*Sesamum indicum*	MT186120	MT186088	MT186136	MT186080	MT338039	MT186104
	VIC 39069	*Commelina benghalensis*	-	KY287250	-	-	KY351634	KY287251

(Continued)

Table 1. (Continued).

Fungal species	Strain number	Host name	GenBank Acc. No.					
			ACT	CAL	GAPDH	HIS3	ITS	tef1
Cercospora tetragoniae	HL T-1	*Tetragonia tetragonoides*	LC579811	LC579812	-	-	-	LC579813
	HL Tt-1	*Tetragonia expansa*	LC589278	LC589277	-	-	MT095118	LC589279
Cercospora violae	**CBS 251.67**	*Viola tricolor*	JX143250	JX143004	MH496322	JX142758	JX143737	JX143496
	CPC 5368	*Viola odorata*	JX143251	JX143005	-	JX142759	JX143738	JX143497
Cercospora zeae-maydis	CBS 117756	*Zea mays*	DQ185097	DQ185109	-	DQ185121	DQ185073	DQ185085
	CBS 117757	*Zea mays*	DQ185098	DQ185110	-	DQ185122	DQ185074	DQ185086
Cercospora zeina	**CPC 11995**	*Zea mays*	DQ185105	DQ185117	-	DQ185129	DQ185081	DQ185093
	CPC 11998	*Zea mays*	DQ185106	DQ185118	-	DQ185130	DQ185082	DQ185094
Cercospora cf. *zinniae*	CBS 132624	*Zinnia elegans*	JX143272	JX143026	-	JX142780	JX143756	JX143518
	CBS 132676	*Zinnia elegans*	JX143273	JX143027	-	JX142781	JX143757	JX143519
Cercospora zebrina	CCTU 1039	*Alhagi camelorum*	KJ886062	KJ885901	MH496323	KJ886223	KJ886545	KJ886384
	CCTU 1185	*Vicia* sp.	KJ886066	KJ885905	MH496333	KJ886227	KJ886549	KJ886388
	CCTU 1012	*Medicago* sp.	KJ886061	KJ885900	MH496328	KJ886222	KJ886544	KJ886383
Septoria provencialis	CBS 118910	*Eucalyptus* sp.	JX143276	JX143030	JX142538	JX142784	DQ303096	JX143522

were harvested and were included in this study. Polystyrene germination boxes were cleaned internally with 70% ethanol and then each lined with two layers of sterile blotter paper and were moistened with sterile water. Seeds were surface disinfected by immersion in 70% alcohol for 1 min, followed by immersion in 1% sodium hypochlorite for 1 min, and then rinsing in sterile tap water. The seeds were then placed within the boxes 1 2 cm spacings, using sterile forceps. An aliquot of a 5 ppm dichlorophenoxyacetate (2,4-D) solution was then added to each box to stop seed germination. The boxes were then maintained for 7 d at 25°C under a 12 h photoperiod. The seeds were then examined under a stereoscopic microscope to assess for presence of fungal conidiophore fascicles and conidia. Confirmation of the identity of the fungi was through observation of morphology, as described above. Fifty seeds from each source were evaluated in this preliminary assessment.

DNA extraction, PCR amplification and sequencing

Representative single conidium isolates of the fungus obtained from necrotic NZ spinach tissues and from beetroot (see Table 1) were grown on PDA (Kasvil) at 25°C under a 12 h photoperiod for 1 week, and genomic DNA was extracted, as described by Duarte *et al*. (2016). The primers ITS4 and ITS5 (White *et al*., 1990) were used to amplify the ITS region and the 5.8S rRNA gene. Additionally, five informative gene fragments were amplified, including actin (*act*), calmodulin (*cal*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), histone3 (*his3*), and translation elongation factor 1-alpha (*tef1-α*), with the respective primer pairs ACT-512F/ACT-783R (Carbone and Kohn, 1999), CAL-228F/CAL2Rd (Carbone and Kohn, 1999), GDF1/GDR1 (Guerber *et al*., 2003), CYLH3F/CYLH3R (Crous *et al*., 2004), and EF1-728F/EF1-986R (Carbone and Kohn, 1999).

PCR products were analyzed on 2% agarose electrophoresis gels stained with GelRed™ (InstantAgarose™) in a 1× TAE buffer, and were visualized under UV light to check for amplification extent and purity. PCR products were purified and sequenced by Macrogen Inc. (http://www.macrogen.com).

Phylogenetic analyses

The resulting nucleotide sequences were edited with the DNA Dragon software (https://www.dna-dragon.com/index.php). All sequences were checked

manually, and nucleotides with ambiguous positions were clarified using primer sequences in both directions. Resulting sequences were deposited in GenBank (www.ncbi.nlm.nih.gov), and are described in Table 1. Sequences obtained from GenBank datasets and the novel sequences generated during this study were aligned using MEGA X (Kumar *et al.*, 2018). Appropriate models were selected for each gene partition using MrModeltest ver. 2.3 (Nylander 2004). Based on the results of MrModeltest, the evolutionary model K80+G was applied to *act*; K80 was used with the ITS partitions; HKY+G was applied to *cal* and *his3* regions; and GTR+G was applied to *gapdh* partition.

Phylogenetic analyses were based on a concatenated dataset of *act*, *cal*, *gapdh*, *his3*, and ITS regions, which were combined using SequenceMatrix (Vaidya *et al.*, 2011). To assess relationships between isolates, two independent algorithms were used: Maximum-Likelihood (ML) and Bayesian inference (BI), both present in the CIPRES web portal (Miller *et al.*, 2010). ML analyses used RAxML v. 8.2.12 (Stamatakis, 2014), and bootstrap values (BS) were determined after 1000 bootstrap samples. BI analyses were performed using MrBayes ver. 3.2.1 (Ronquist *et al.*, 2012) and applying the substitution models listed above. The Markov chain Monte Carlo (MCMC) method was used to search for the best tree topology. Two simultaneous and independent analyses were performed, each with four chains. MCMCs were run for 5,000,000 generations, and trees were sampled every 500th generation, until convergence was reached. The first 25% of trees were discarded as the burn-in phase. The remaining 7,500 trees from each run generated the consensus tree, from which posterior probabilities values (PPs) were obtained.

The resulting trees were visualized in FigTree (Rambaut, 2012). ML and BI topologies were compared, and the BI topology was adopted. The BI tree was exported to graphic software, and BS values greater than 70%, or PP values greater than 0.95, were maintained. *Septoria provencialis* (isolate CBS 118910) served as the outgroup for the phylogenetic analyses.

Pathogenicity tests

Inocula of isolates COAD 2380 (obtained from NZ spinach) and COAD 2476 (from beetroot) were cultivated using the "biphasic method" (Jackson *et al.*, 1996) with modifications. Aliquots (100 mL each) of potato dextrose (PD) were placed in separate 250 mL capacity flasks, and were then autoclaved for 20 min at 121°C. After cooling, each flask was seeded with five 1 cm diam. disks obtained from the margin of an actively growing PDA colony of one of the isolates. The flasks were then placed on an orbital shaker (Marconi®-MA420) set at 130 rpm and 25 +/- 2°C, and then incubated for 30 d. The flasks were drained and the mycelium in each was separated. The mycelium masses were suspended in sterile water, triturated with a mortar, and then transferred onto potato carrot agar (PCA; Johnston and Booth, 1983) in Petri plates. The plates were then incubated under the conditions described above. After 14 d, the surface of each plate was flooded with 10 mL of sterile water, scraped with a rubber spatula, and the resulting material was filtered through cheesecloth. The resulting conidium suspensions were adjusted to 1.4×10^6 conidia mL^{-1}.

Four one-month-old healthy NZ spinach plants, grown from seeds (Isla Sementes) in separate 2 L capacity pots containing a mixture of pasteurized soil and manure, were used in pathogenicity tests. Two plants were sprayed until runoff with the conidium suspension and two plants were sprayed with sterile tap water as inoculation controls.

Additionally, two healthy 2-month-old beetroot plants were also inoculated with isolate COAD 2380 conidium suspension, and two NZ spinach plants were inoculated with isolate COAD 2476. All the plants were then left in a dew chamber for 48 h, then transferred to a greenhouse bench, where they were observed each day for disease symptoms.

RESULTS AND DISCUSSION

Molecular identification of isolates

Phylogenetic studies combining *act*, *cal*, *gapdh*, *his3*, and ITS regions were based on 50 *Cercospora* taxa and the outgroup *Septoria provencialis*. The combined alignment comprised 1846 characters with gaps (187 for *act*, 237 for *cal*, 686 for *gapdh*, 306 for *his3*, and 430 for ITS). Previous studies have shown that it is important to include the *cal* and *gapdh* regions in analyses of such *Cercospora* taxa (Groenewald *et al.*, 2013; Bakhshi and Zare, 2020). The combined data obtained in the present study confirmed this.

Phylogenetic analyses indicated that the *Cercospora* isolates obtained from *T. tetragonoides* (isolates COAD 2380 and COAD 2477) and *B. vulgaris* (isolates COAD 2476 and COAD 2478) formed a monophyletic and well supported clade with *C. beticola* (BS/PP = 96/1) (Figure 1). The clade containing the isolates under study, as well as the *C. beticola* isolates, was separated from *C. apii*, *C. apiicola*, *C. asparagi*, and *C. gamsiana* (Figure 1). These results demonstrate that the fungus from NZ spinach examined in this study was *C. beticola*.

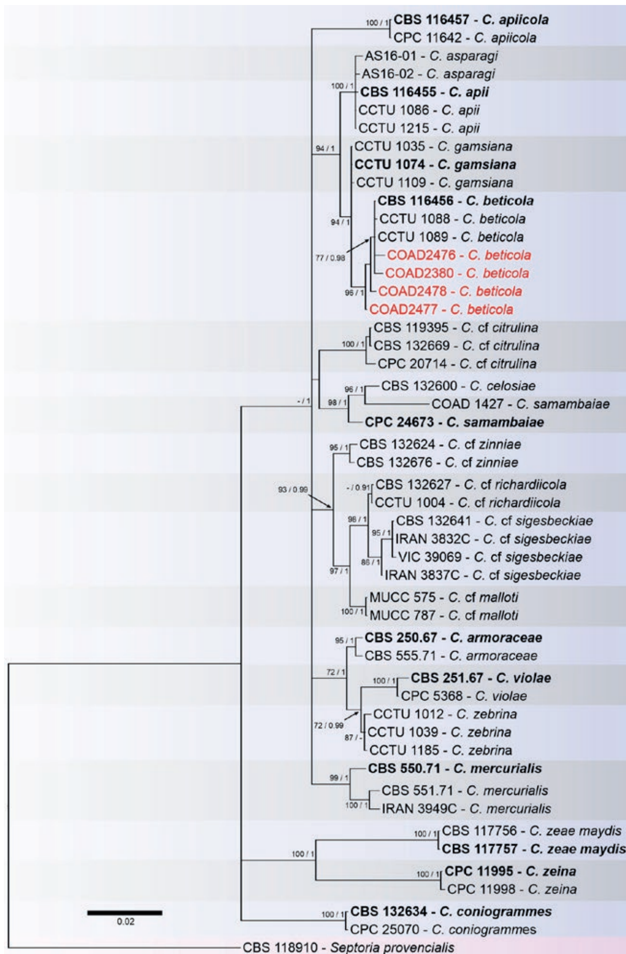

Figure 1. Consensus tree of selected *Cercospora* species, with topology from Bayesian analysis of the combined *act*, *cal*, *gapdh*, *his3*, and ITS regions. Numbers before and after slashes, respectively, represent likelihood bootstrap and posterior probabilities values. The tree is rooted with *Septoria provencialis* (isolate CBS118910). Isolates collected and included in this study are in red font, and ex-type isolates are in bold font. Scale bar indicates 0.02 expected changes per site.

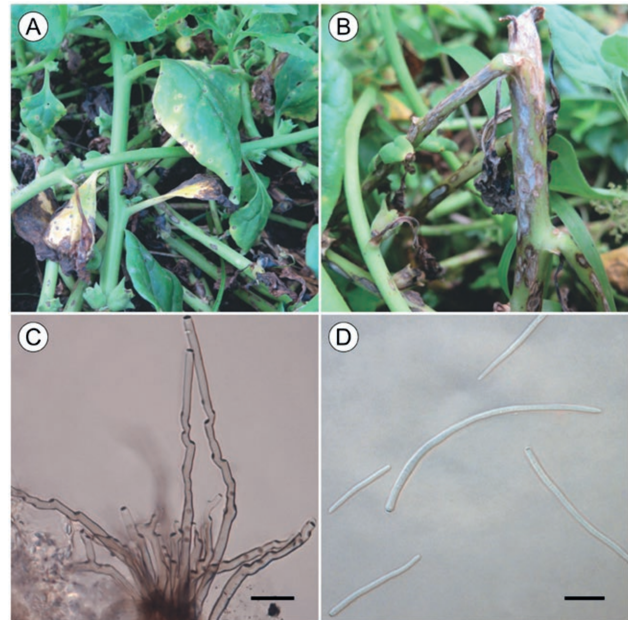

Figure 2. *Cercospora beticola* on leaves and stems of *Tetragonia tetragonoides*. A, leaf spots. B, stem spots. C, geniculate conidiophores with conspicuous conidiogenous loci. D, subcylindrical hyaline pluriseptate conidia with thickened and darkened scars. Scale bars = 30 µm.

Taxonomy

Morphology of the fungus on *T. tetragonoides* was recognized at early stages of this study as typical of the broad assemblage of fungi placed by Crous and Braun (2003) in *Cercospora apii sensu lato*, a group including *C. beticola.*

Cercospora beticola Sacc., *Nuovo Giornale Botanico Italiano* 8 (2): 189 (1876), Fig. 2 A-D.

Symptoms. Leaf lesions starting as small dark brown dots, circular becoming irregular to sub-circular on leaves and elongated on stems, white to grey, each centrally, surrounded by a dark brown rim, 1–3 mm diam., later coalescing and leading to yellowing of leaves, causing premature defoliation; on stems necrotic lesions similar to those on leaves (but somewhat elongated), progressively girdling the stems and causing branch dieback (Figure 2, A and B).

Morphology. Mycelium intra- and intercellular, hyphae branched, septate, pale brown, 2–5 µm wide. Stromata sub-epidermal, irregular, 13–40 × 13–38 µm, and dark brown. Conidiophores cylindrical, fasciculate, 65–162 × 2–5 µm, 2–6 septate, grey-brown at the bases, becoming paler towards the apices, smooth. Conidiogenous cells terminal, integrated, cylindrical, 7–20 × 2–5 µm, pale brown, smooth. Conidiogenous loci conspicuous, 1 to 2 per cell, 2–3 µm diam., thickened and darkened. Conidia solitary, acicular to sub-cylindrical, straight to slightly curved, or sinuous, hyaline, smooth, 55–252 × 2.5 µm, 6–32 septate, each attenuating from base towards the subacute tip, sub-truncate at the base, with a thickened and darkened hilum.

In culture. PDA colonies slow-growing (2 cm diam. after 14 d), flat, cottony, dense and smoke grey centrally, sparser and grey olivaceous towards the periphery, with irregular borders, and olivaceous black reverse sides; not sporulating.

Material examined. Brazil: Minas Gerais, Viçosa, on *Tetragonia tetragonoides*, 10 November 2017, G. Kolesza (VIC 44406, culture COAD 2380).

Additional material. Brazil: Minas Gerais, Viçosa, on leaves of *Beta vulgaris*, 26 April 2018, G. Kolesza (VIC 44456, culture COAD 2476); Rio de Janeiro, Petrópolis, Bonfim, on *Tetragonia tetragonoides*, 16 April 2018, R. W. Barreto (VIC 44457, culture COAD 2477); Rio de Janeiro, Petrópolis, Bonfim, on leaves of *Beta vulgaris*, 16 April 2018, R. W. Barreto (VIC 44458, culture COAD 2478).

At 13 d after inoculation, typical symptoms equivalent to those observed in the field appeared on the two inoculated plants of NZ spinach, and on the beetroot plants, but not on the inoculation control plants. Conidiophores, fascicles and conidia of *Cercospora beticola* were present on the necrotic tissues. A fungus was reisolated from diseased tissues, and colonies obtained were identical to those of the inoculated fungus originally obtained from NZ spinach. The cross-inoculations of COAD 2380 and COAD 2476 resulted in typical *Cercospora* leaf spot symptoms, both on NZ spinach (Figure 3, A and B) and beetroot (Figure 3, C and D).

Some conidiophore fascicles of *Cercospora beticola*, each bearing abundant acicular conidia, were present in all of the examined NZ spinach seed lots, including on freshly collected seeds from the plots where the disease was first observed. Incidence of the fungus on seeds was small, ranging from two to four seeds per batch of 50 seeds. This was confirmation of the earlier assessments of Hino and Tokeshi (1978), and justifies further studies on the potential for dissemination of this important disease through infected and marketed seeds (fruits).

Since Spegazzini's first description of the fungus on NZ spinach as *Cercosporina tetragoniae*. based on a specimen collected in La Plata (Argentina), and Siemaszko's recombination into *Cercospora,* this fungus has been examined by experts on the taxonomy of *Cercospora* and allied fungi. As Siemaszko's recombination appeared in an obscure publication, it escaped Chupp's (1954) monograph. Chupp went on to propose the superfluous recombination *C. tetragoniae*. The holotype material, deposited at LPS (Fungarium Instituto Spegazzini, La Plata), was re-examined by Chupp (1954), Sutton and Pons (1980), and Braun (2000). Although these authors recognized the type material as being scarce, they found some conidiophores and conidia on it and confirmed the identity of *C. tetragoniae* as a member of *Cercospora*. Braun (2000) emphasized that this species is indistinguishable from *C. apii sensu lato*, which is a broad morphological concept proposed by Crous and Braun (2003) which included *C. beticola*. Crous and Braun (2003) introduced the concept of "compound species" which each consisted of morphologically indistinguishable species with different races (host ranges), that were genetically uniform or heterogeneous, with different degrees of biological specialization. They also proposed that genetically and morphologically clearly distinguishable taxa should be treated as separate species. Crous and Braun (2003) proposed that *C. tetragoniae* should be regarded as a synonym of *C. apii*. *Cercospora* species on NZ spinach were not included in the later, critical publication by Groenewald et al. (2005), which led to re-establishment of *C. beticola*. In 2015, the name *C. tetragoniae* reappeared in the literature, along with description and illustration of the fungus based on holotype, but with no mention of the earlier proposal of this to be regarded as a synonym of *C. apii sensu lato* (Braun *et al.,* 2015). The present authors agree with Braun (2000), and also consider *C. tetragoniae* indistinguishable from *C. apii sensu lato* (the assemblage containing *C. beticola*). Fungus morphology and host symptoms both indicate that *C. tetragoniae* is a synonym of *C. beticola*. Nevertheless, for final clarification of this nomenclatural issue, the fungus should be recollected from the type locality of *C. tetragoniae* in La Plata for definitive molecular studies.

Figure 3. Results from cross-inoculation tests between *Cercospora beticola* isolates obtained from *Tetragonia tetragonoides* (COAD 2380) and beetroot (COAD 2476). A and B, *T. tetragonoides* plants inoculated with *C. beticola* isolate COAD 2476, and C and D, beetroot plants inoculated with *C. beticola* COAD 2380, after 2–3 weeks from inoculations.

Cercospora beticola is a broad-spectrum pathogen attacking 42 host species in 20 genera of several plant families (Crous and Braun, 2003), including *Acanthaceae*, *Apiaceae*, *Amaranthaceae*, *Asteraceae*, *Plumbaginaceae*, *Rosaceae*, *Malvaceae*, *Plantaginaceae*, *Polygonaceae*, *Martyniaceae*, *Pedaliaceae* and *Solanaceae* (Farr and Rossman, 2021). *Cercospora beticola* is known as the etiological agent of the most important foliar disease of beetroot (Tedford *et al*., 2018), and regarded as the most important disease of beetroot in Brazil (Carmelo-Gacia *et al*., 2016). This pathogen also causes severe leaf spot of Swiss chard (Soylu *et al*., 2003), a form of *Beta vulgaris*, and of spinach (*Spinacia oleracea*) (Mukhtar *et*

al., 2019). NZ spinach is likely to be an additional host of the broad host ranged *C. beticola*.

Despite *C. tetragoniae* being mentioned in previous reports, no molecular information linked to publications on this species is available. The lack of molecular data from *C. tetragoniae* led us to attempt to obtain the topotypic material of this fungus, but without success despite several attempts. Since the early 1900s, interest by vegetable growers of La Plata and cool areas of Argentina in production of NZ spinach has vanished.

Searches for *C. tetragoniae* in the NCBI nucleotides database identified sequences associated with two isolates listed as *C. tetragoniae*. These isolates were listed as obtained from *T. tetragonoides* and *T. expansa* (a synonym of *T. tetragonoides*), and were referred to as part of a study to be published in the future, which would report the occurrence of leaf spots caused by *C. tetragoniae* on *T. tetragonoides* in Taiwan. When incorporated into the present study phylogenetic analysis, these *C. tetragoniae* isolates formed a well-supported and distinct clade from the present study isolates (Figure 1). It is not clear whether the Taiwanese isolates represent "true *C. tetragoniae*" until further information on these isolates becomes available.

There is no previous record of *C. beticola* affecting NZ spinach, other *Tetragonia* spp., or any other member of the *Aizoaceae*. Records of *Cercospora* (either as *Cercospora* sp. or *Cercospora tetragoniae*) on *T. tetragonoides* (or its synonym *Tetragonia expansa*) in Farr and Rossman (2021), and the New Zealand list of fungi (Landcare, 2020), among other databases, are based on herbarium records or names appearing in pathogen lists, which are not accompanied by taxonomic or phytopathological information. There are numerous published records of *C. tetragoniae* on *T. tetragonoides* [= *T. expansa*] from Africa, Asia, South, Central and North America, listed in Braun *et al.* (2015). Strangely the authors of these records have ignored the previous view of Braun (2000) that *C. tetragoniae* was a late synonym of *C. apii*. In New Zealand, *C. tetragoniae* was collected for the first time in 2008 on NZ spinach (Landcare, 2018). *Cercospora beticola* had been recorded much earlier from New Zealand, but on Swiss chard (Dingley, 1969) and beetroot (Pennycook, 1989).

The results of the cross-inoculation study performed here, involving one NZ spinach isolate (COAD 2380) and one beetroot isolate (COAD 2476), confirmed that NZ spinach can be a host for *C. beticola*, and that one crop host may serve as the inoculum source for disease outbreaks on the other. This could be of relevance for crop management, since both crops are often cultivated in the same vegetable gardens or in neighboring areas.

Although the present study is preliminary and prospective, demonstration of occurrence of the leaf spot pathogen of NZ spinach in 'seeds' deserves further investigation in Brazil and elsewhere. Pittner *et al.* (2016), showed that *C. beticola* impaired beetroot seed quality, leading to loss of viability of 'seeds', and poor germination and emergence after sowing, and contributed to disseminating the pathogen over long distances. It is likely that the same applies to this fungus on NZ spinach.

Considering the relevance of NZ spinach as an internationally important vegetable crop, broader surveys should be carried out, including isolation and characterization of *Cercospora*-like fungi associated with leaf spots on NZ spinach in other countries. These would further clarify the relevance of *C. beticola* as a pathogen for this crop, and clarify the diversity of cercosporoid species associated with NZ spinach.

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Review

Current status of *Botryosphaeriaceae* species in Italy: Impacts on agricultural crops and forest ecosystems

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Summary. Many fungi belonging to *Botryosphaeriaceae* are well-known as causal agents of diseases in economically and ecologically important agricultural crops and forest trees. In Italy, the high diffusion of *Botryosphaeriaceae* infections observed over the last decade, has shown the importance of this group of fungi, which are becoming limiting factors for plant production in agricultural systems, nurseries and natural and urban landscapes. Global warming and stress factors such as occasional extreme climatic events can affect the susceptibility of host plants, as well as fungus behaviour, increasing the risk of future infections. Available reports of *Botryosphaeriaceae* in Italy have been examined, focusing on wood and fruit pathogens, resulting in a list of ten genera and 57 species. *Diplodia* is the most widespread genus in Italy with 76 records on 44 hosts, while at species level, *Neofusicoccum parvum*, *Botryosphaeria dothidea* and *Diplodia seriata* show the widest host ranges and many records. The ability of the pathogens to remain latent on asymptomatic plants, and uncontrolled trade of plant materials among countries, facilitate the dissemination and potential introduction of new *Botryosphaeriaceae* species. Preventive detection and adequate control strategies are always needed to limit the potential damage caused by *Botryosphaeriaceae*. This review had particular emphasis on host-pathogen associations, disease symptoms, geographic distribution, metabolite production, and accurate pathogen identification.

Keywords. Geographic distribution, host-range and disease symptoms, invasive pathogens, metabolites production, species identification.

INTRODUCTION

Botryosphaeriaceae Theiss. & Syd. is one of the most investigated families of fungi (Agnoletti *et al.,* 2022). In addition to including primary plant pathogens such as *Diplodia corticola* and *Lasiodiplodia theobromae*, *Botryosphaeriaceae* includes some species that can live as endophytes in healthy plants or as saprophytes on dead host tissues (Alberti *et al*., 2018; Aiello *et al*., 2020, 2022). Recent interest in this family has been linked to the abilities to survive as latent endophytes and to change to pathogenic behaviour when host plants are under stress conditions. Many fungi belonging to *Botryosphaeriaceae* may cause severe diseases of woody plants in natural and urban areas, nurseries and in agricultural crops (Slippers and Wingfield, 2007; Linaldeddu *et al.,* 2016a; Mehl *et al.,* 2017; Zlatković *et al*., 2017; Aiello *et al*., 2020; Guarnaccia *et al*., 2016, 2023). Some of these fungi can also be found on important agricultural non-woody crops.

The global spread of these fungi occurs through the international movement of plants and derivatives without appropriate quarantine systems, while short-distance spread is mainly due to spores carried by rain, wind and, less so, via insects (Van Niekerk *et al.,* 2006; Moyo *et al.,* 2014; Valencia *et al.,* 2015; Panzavolta *et al.,* 2018; Pinna *et al.,* 2019). Diseases caused by *Botryosphaeriaceae* can be mono- or oligo-cyclic (undergoing two to three infection cycles per season), and epidemic events may occur for subsequent years, resulting in high economic losses. These fungi can also spread from nurseries to open fields as latent infections (Moral *et al.,* 2019).

Prior to the mid-1990s, most *Botryosphaeriaceae* species were identified based on micro- and macro-morphological characters. In the recent years, research on *Botryosphaeriaceae* diseases has extended on many crops and required increasingly efficient identification tools, especially due increased recognition and awareness that these fungi are important wood and fruit rot pathogens. Advances in molecular DNA molecular methods have provided reliable tools to discriminate cryptic species, accommodate or synonymize some *taxa*, and describe new genera and species. Currently, the family includes 22 genera and 281 species and some putative hybrids such as those found in *Lasiodiplodia* (Crous *et al*., 2006; Liu *et al.,* 2012; Phillips *et al.,* 2013; Dissanayake *et al.,* 2016a; Linaldeddu *et al.,* 2016a; Slippers *et al.,* 2017; Zhang *et al.,* 2021).

Climate is considered a major factor affecting the geographical distribution of *Botryosphaeriaceae* species. Some have a limited distribution, whereas a few species such as *Botryosphaeria dothidea*, *D. sapinea*, *D. seriata*, *Dothiorella sarmentorumm*, *L. theobromae* and *Neofusicoccum parvum*, are distributed much more widely (Batista *et al.,* 2021). It is possible to predict the occurrence of *Botryosphaeriaceae* species in space and time, and to evaluate the potential for their spread over time, using Species Distribution Models (SDMs) (Batista *et al.,* 2023). As a consequence of global warming and climate change some species could shift their ecological ranges, such as *B. dothidea* that could spread in the northern hemisphere, or *N. parvum* for which a change in its latitudinal range is expected. Otherwise, for species such as *L. theobromae*, future scenarios predict diffusion within tropical and sub-tropical regions (Batista *et al.,* 2023). In Italy, *D. sapinea* is mostly widespread in central and southern areas on pine forests. However, the future climate scenario foresees a 9 to 40% increase in its infection habitat, mainly in response rises in mean temperature of the wettest and driest areas (Bosso *et al.,* 2017).

Differences in host susceptibility, pathogen virulence and environmental factors have significant effects on disease development caused by *Botryosphaeriaceae* (Zwolinski *et al.,* 1990; Swart and Wingfield, 1991; Johnson *et al.,* 1997). Stress factors such as occasional climatic events can affect host susceptibility and pathogen behaviour, increasing the risk of infections. Drought or heat stress can negatively impact plant physiology, enhancing pathogen colonization and increasing host susceptibility (Batista *et al.,* 2021). Swart and Wingfield, (1991) reported that water stress, pruning and hail injury could promote *D. sapinea* infections on *Pinus* species. However, drought affects the disease only in aggressive pathogen strains (Blodgett and Stanosz, 1997). Pathogens such as *B. dothidea* (Ma *et al.,* 2001) and *D. mutila* (Ragazzi *et al.,* 1999) have been reported to infect water stressed hosts. These observations were also confirmed for other *Botryosphaeriaceae* pathogens, such as *N. australe*, *N. parvum*, *L. theobromae* and *D. seriata* (Van Niekerk *et al.,* 2011a, 2011b).

As for many pathogens, *Botryosphaeriaceae* use virulence factors to overcome plant defences and facilitate adhesion to hosts, as well as to facilitate colonization in the initial stage of infections (Sacristán and García-Arenal, 2008; Tan and Liang, 2013). Next-generation sequencing techniques have demonstrated that different gene classes are involved in *Botryosphaeriaceae* pathogenesis, toxins and other secondary metabolites are known to have phytotoxic effect on plants, and wood degradation enzymes may cause some disease symptoms (Belair *et al*., 2022). Grapevine foliar symptoms caused by fungal wood pathogens are usually associated with phytotoxic metabolites produced during wood colonization (Masi *et al*., 2018a, 2018b). Some metabolites have activity against many fungal pathogens, such as com-

pounds produced by *D. corticola* and *D. subglobosa* that could offer numerous benefits in multiple biotechnology sectors (Cimmino *et al.*, 2016; Masi *et al.*, 2022), and occurrence of typical chloro-necrotic foliar symptoms on grapevine have been associated with infections by *Botryosphaeriaceae* species (Dubos *et al.*, 2001; Abou-Mansour *et al.*, 2015).

In Italy, the increasing number of reports of *Botryosphaeriaceae* infections in different agricultural crops and urban and natural ecosystems have shown the importance of this group of fungi, which are now recognized as limiting factors for plant production. The aim of the present review is to analyze recent advances in knowledge of *Botryosphaeriaceae* that cause wood infections, decline and/or fruit damage in Italy, with particular emphasis on new host-pathogen interactions, geographic distribution, disease symptoms, production of metabolites and aspects related to accurate pathogen identification.

SPECIES AND GEOGRAPHIC DISTRIBUTION OF *BOTRYOSPHAERIACEAE* IN ITALY

Although severe diseases caused by *Botryosphaeriaceae* have been well-known for a long time in Italy, these pathosystems have only been investigated in detail during the last 20 years.

Based on available reports that include sequence data, ten genera and 57 species of *Botryosphaeriaceae* have been reported in Italy, with 271 host-pathogen associations in the agricultural, horticulture and forestry (Table 1). The distributions of pathogen species and genera among Italian regions is irregular, with most reports from intensely cultivated areas and Mediterranean forest ecosystems dominated by native shrubs and trees (Table 1). The distribution of pathogen species is not associated to a phylogeographic patterns, although some fungal genera are more common in mountain areas (e.g. *Dothiorella* and *Neofusicoccum*) and others (*Lasiodiplodia*) in the warmest areas of southern Italy. In particular, Sardinia (26 species) and Sicily (20 species) are the regions with the greatest numbers of reported species (Table 1). The few records from some regions probably reflects the limited sampling efforts, distributions of plant hosts, and levels of susceptibility.

Some polyphagous species, such as *B. dothidea*, *D. seriata* and *N. parvum*, have large/wide geographic distributions. On the other hand, other species that infect only a few host plants are restricted to small geographic areas, and these pathogens include *B. auasmontanum*, *D. insularis* and *Sardiniella urbana* (Table 1).

Overall, 45 plant host families, 94 genera and 130 species of native and exotic plants have been recorded as susceptible to *Botryosphaeriaceae* species in Italy (Table 1). Commonly affected host genera include *Fraxinus, Olea, Quercus*, and *Vitis*. On *Quercus* and *Fraxinus* spp., *D. corticola* and *D. fraxini* are reported as the main pathogens involved in the complex aetiology of, respectively, oak and ash decline (Linaldeddu *et al.*, 2014, 2020b). In addition to these two key species, many other species of *Botryosphaeriaceae*, such as *B. dothidea, D. seriata, D. subglobosa, Do. iberica* and *N. parvum*, have been isolated from symptomatic oak and ash tree tissues (Moricca *et al.*, 2012; Linaldeddu *et al.*, 2014; Moricca *et al.*, 2016; Linaldeddu *et al.*, 2020b).

Besides damaging natural ecosystems, *Botryosphaeriaceae* are important pathogens of many traditional and emerging agricultural crops, such as avocado, fig, grapevine, hazelnut, lemon, loquat, mango, olive, orange, pistachio, pomegranate and walnut (Lazzizera *et al.*, 2008a; Ismail *et al.*, 2013; Carlucci *et al.*, 2015; Linaldeddu *et al.*, 2015a, 2016b, 2020a; Giambra *et al.*, 2016; Aloi *et al.*, 2021; Aiello *et al.*, 2020, 2022; Gusella *et al.*, 2021, 2022, 2023a). Several aggressive species are involved in these pathosystems, including *B. dothidea*, *D. olivarum, L. mediterranea* and *N. parvum*. Grapevine and olive are susceptible to a large number of *Botryosphaeriaceae* species responsible for diverse symptoms, including cankers, dieback and fruit rots. Out of 46 species of *Botryosphaeriaceae* reported on grapevines worldwide, 19 have been reported in Italy (Table 1), whereas *Macrophomina phaseolina* is the main causal agent reported on herbaceous plants (Faedda *et al.*, 2016).

IMPACTS OF DISEASES CAUSED BY *BOTRYOSPHAERIACEAE* SPECIES

This section outlines reports of host ranges and symptoms caused by *Botryosphaeriaceae* on ecologically and economically important forestry and agricultural plants.

Very few species of *Botryosphaeriaceae* are host specific. Most are polyphagous and can potentially cause infections on a broad range of crops under particular conditions (neutral host behaviour). *Botryosphaeriaceae* can also infect native hosts, and then move to other introduced hosts in the same region (Pavlic *et al.*, 2007; Luo *et al.*, 2022). *Botryosphaeriaceae* are responsible for cankers on host trunks, branches and twigs, dieback and shoot blight, bark cracking, wood discolouration, stem-end rots and fruit rots (Carlucci *et al.*, 2015; Aiello *et*

Table 1. Species of *Botryosphaeriaceae* and their hosts reported in Italy.

Species	Host	Symptoms	Region	Reference
Botryosphaeria dothidea	*Acer pseudoplatanus* *Ailanthus altissima* *Artemisia* sp. *Carpinus betulus* *Clinopodium nepeta* *Colutea arborescens* *Colutea cilicica* *Cornus sanguinea* *Cydonia oblonga* *Euonymus europaeus* *Ficus microcarpa* *Fraxinus excelsior* *F. ornus* *Galium* sp. *Juglans regia* *Laburnum* sp. *Malus domestica* *Mangifera indica* *Micromeria graeca* *Olea europaea* *Ostrya carpinifolia* *Persea americana* *Phaseolus vulgaris* *Pistacia vera* *Populus tremula* *Prunus armeniaca* *Pseudotsuga menziesii* *Punica granatum* *Pyrus communis* *Quercus ilex* *Quercus robur* *Quercus rubra* *Quercus suber* *Sambucus ebulus* *Sambucus nigra* *Salix* sp. *Torilis arvensis* *Urtica dioica* *Vitis vinifera*	Cankers, branch dieback, wood necrosis, shoot blight, fruit rot	Apulia, Basilicata, Campania, Emilia Romagna, Friuli Venezia Giulia, Lazio, Lombardy, Molise, Piedmont, Sicily, Sardinia, Tuscany, Veneto	Aiello *et al*., 2022 Bertetti *et al*., 2013 Carlucci *et al.,* 2013, 2015 Dardani *et al*., 2023 De Corato *et al*., 2007 Dell'Olmo *et al*., 2023 Dissanayake *et al.,* 2017 Fiorenza *et al*., 2022, 2023 Garibaldi *et al.,* 2012 Grasso and Granata, 2010 Gusella *et al*., 2021, 2022 Lazzizera *et al.,* 2008b Li *et al*., 2020 Linaldeddu *et al*., 2009, 2014, 2015a, 2020a, 2020b Marinelli *et al*., 2012 Martino *et al*., 2023 Moricca *et al.,* 2008 Piskur *et al*., 2011 Raimondo *et al*., 2019 Scala *et al*., 2019 Schlegel *et al*., 2018 Spagnolo *et al*., 2011 Turco *et al*., 2006 Wijesinghe *et al*., 2021 Zimowska *et al*., 2020
syn. *B. auasmontanum*	*Alnus cordata* *Rosa canina*	Shoot blight, cankers and dieback	Emilia Romagna	Dissanayake *et al.,* 2017
Diplodia africana	*Grevillea robusta* *Juniperus oxycedrus* *Juniperus phoenicea* *Pinus nigra* *Pinus pinea* *Quercus ilex* *Vitis vinifera*	Cankers, dieback	Campania, Sardinia, Sicily	Cristinzio *et al*., 2015 Giambra *et al*., 2019 Linaldeddu *et al*., 2011a, 2015a Luchi *et al*., 2014 Seddaiu *et al*., 2019
D. corticola	*Quercus coccifera* *Quercus ilex* *Quercus pubescens* *Quercus suber* *Vitis vinifera*	Sunken and bleeding cankers, dieback, wood necrosis, V-shaped necrotic sectors	Apulia, Molise, Sardinia, Sicily, Tuscany	Carlucci *et al*., 2015 Carlucci and Frisullo, 2009 Linaldeddu *et al*., 2011b, 2013, 2014 Raimondo *et al*., 2019
D. crataegicola	*Crataegus* sp. *Prunus* sp. *Tilia* sp.	Cankers and branch dieback	Emilia Romagna	Ariyawansa *et al*., 2015 Dissanayake *et al.,* 2017

(Continued)

Table 1. (Continued).

Species	Host	Symptoms	Region	Reference
D. cupressi	*Cupressus sempervirens** *Pinus nigra*	Cankers, dieback, V-shaped necrotic sectors, shoot blight	Abruzzo, Calabria, Sardinia*	Luchi *et al.*, 2014 This study*
D. fraxini	*Fraxinus angustifolia* *F. excelsior*	Cankers, bark discoloration, dieback, V-shaped necrotic sectors	Veneto, Friuli Venezia Giulia	Alves *et al.*, 2014 Linaldeddu *et al.*, 2020b
D. pseudoseriata	*Fraxinus angustifolia*	-	Sardinia	Alves *et al.*, 2014
syn. *D. insularis*	*Pistacia lentiscus*	Leaf chlorosis, crown thinning, V -shaped necrotic sectors branch dieback, sunken cankers	Sardinia	Linaldeddu *et al.*, 2016c
syn. *D. alatafructa*	*Picea abies*	Cone necrosis	Emilia Romagna	Dissanayake *et al.,* 2017
D. malorum	*Populus alba*	-	Sardinia	Alves *et al.*, 2014
D. mutila	*Acer negundo* *Colutea arborescens* *Fraxinus excelsior* *Olea europaea* *Phaseolus vulgare* *Populus tremula* *Vitis vinifera*	Cankers, wood necrosis	Apulia, Campania, Emilia Romagna, Friuli Venezia Giulia, Molise, Piedmont, Sardinia, Veneto	Carlucci *et al.*, 2013, 2015 Alves *et al.*, 2014 Dardani *et al.*, 2023 Dissanayake *et al.,* 2017 Linaldeddu *et al.*, 2015a, 2020b Liu *et al.*, 2015 Raimondo *et al.*, 2019 Dell'Olmo *et al.*, 2023
D. olivarum	*Ceratonia siliqua* *Olea oleaster* *Olea europaea* *Pistacia lentiscus* *Vitis vinifera*	Leaf chlorosis, crown thinning, branch dieback, sunken and bleeding cankers, fruit rot	Sardinia, Sicily, Apulia	Alves *et al.*, 2014 Granata *et al.,* 2011 Lazzizera *et al.,* 2008a Linaldeddu *et al.*, 2015a, 2016c Manca *et al.*, 2020
D. scrobiculata	*Arbutus unedo* *Olea europaea* *Pinus radiata*	Cankers and branch dieback	Apulia, Sardinia	Lazzizera *et al.,* 2008a Linaldeddu *et al.*, 2006a, 2010 Zhang *et al.*, 2020
D. seriata	*Cornus sanguinea* *Corylus avellana* *Cupressus sempervirens* *Eriobotrya japonica* *Euonymus europaeus* *Fraxinus angustifolia* *F. excelsior* *Galium* sp. *Grevillea robusta* *Magnolia grandiflora* *Malus domestica* *Olea europaea* *Pinus nigra* *Pinus sylvestris* *Populus nigra* *Quercus pubescens* *Prunus laurocerasus* *Quercus ilex* *Quercus suber* *Rosa canina* *Sambucus nigra* *Ulmus minor* *Vitis vinifera*	Cankers, branch dieback, wood necrosis, shoot blight, leaf necrosis, fruit rot	Apulia, Emilia Romagna, Friuli Venezia Giulia, Molise, Piedmont, Sardinia, Sicily, Tuscany, Umbria, Veneto	Alves *et al.*, 2014 Ariyawansa *et al.*, 2015 Carlucci *et al.*, 2013, 2015 Dardani *et al.*, 2023 Dissanayake *et al.,* 2017 Giambra *et al.*, 2016, 2019 Lazzizera *et al.*, 2008a Linaldeddu *et al.*, 2006b, 2013, 2014, 2015a, 2016c, 2020b Lorenzini and Zapparoli, 2018 Luchi *et al.*, 2014 Martino *et al.*, 2023 Mondello *et al.*, 2013 Quaglia *et al.,* 2014 Raimondo *et al.*, 2019 Spagnolo *et al.,* 2011 Wijayawardene *et al.*, 2016

(Continued)

Table 1. (Continued).

Species	Host	Symptoms	Region	Reference
D. sapinea	*Cedrus deodara* *Corylus avellana* *Cupressus sempervirens* *Olea europaea* *Picea abies* *Pinus halepensis* *Pinus nigra* *Pinus pinaster* *Pinus pinea* *Pinus radiata* *Pinus sylvestris*	Cankers, branch dieback, Cone necrosis, needle and shoot pine blight	Apulia, Basilicata, Calabria, Campania, Emilia Romagna, Friuli Venezia Giulia, Lazio, Lombardy, Marche, Molise, Piedmont, Sicily, Sardinia, Tuscany, Trentino Alto Adige, Umbria, Veneto	Cabras *et al*., 2006 Maresi *et al*., 2007 Dissanayake *et al.,* 2017 Lazzizera *et al.,* 2008a Linaldeddu *et al*., 2016b Luchi *et al*., 2014
syn. *D. rosacearum*	*Eriobotrya japonica*	Cankers	Sicily	Giambra *et al*., 2016
syn. *D. italica*	*Crataegus* sp.	Canker, branch dieback	Tuscany	Wijayawardene *et al*., 2016 Wijesinghe *et al*., 2021
D. subglobosa	*Fraxinus excelsior* *F. ornus*	Cankers, bark discoloration, dieback, V-shaped necrotic sectors	Veneto, Friuli Venezia Giulia, Sicily	Alves *et al*., 2014 Linaldeddu *et al*., 2020b
Dothiorella eriobotryae	*Rhamnus alaternus* *Tamarix gallica*	Bleeding cankers, dieback	Emilia Romagna	Dissanayake *et al.,* 2017
Do. franceschinii	*Rhamnus alaternus*	Bleeding cankers, dieback	Sardinia	Senanayake *et al.,* 2023
Do. guttulata	*Alnus* sp.	-		Tian *et al*., 2018
Do. iberica	*Acer opalus* *Corylus avellana* *Pinus nigra* *Quercus cerris* *Q. suber* *Rosa canina* *Vitis vinifera*	Cankers and branch dieback	Apulia, Emilia Romagna, Sardinia, Tuscany, Umbria	Carlucci *et al*., 2015 Dissanayake *et al.,* 2016b Linaldeddu *et al*., 2011b, 2016c Luchi *et al*., 2014 Pavlic-Zupanc *et al*., 2015 Phillips *et al.,* 2005 Wijayawardene *et al*., 2016
Do. iranica	*Paliurus* sp.	-	Emilia Romagna	Dissanayake *et al.,* 2016b
Do. omnivora	*Cornus sanguinea* *Corylus avellane* *Fraxinus excelsior*	Cankers, branch dieback	Emilia Romagna, Friuli Venezia Giulia, Veneto	Dissanayake *et al.,* 2017 Linaldeddu *et al*., 2016b, 2020b
Do. parva	*Corylus avellana* *Ostrya carpinifolia*	Cankers and branch dieback	Friuli Venezia Giulia, Sardinia, Veneto	Linaldeddu *et al*., 2016b, 2020b Pavlic-Zupanc *et al*., 2015 Scala *et al*., 2019
Do. sarmentorum	*Clematis vitalba* *Coronilla emerus* *Crataegus* sp. *Hippocrepis emerus* *Paliurus spina-christi,* *Prunus dulcis* *Olea oleaster* *Ulmus minor* *Pinus nigra* *Robinia pseudoacacia* *Ulmus minor* *Vitis vinifera*	Cankers, dieback, pine shoot blight	Apulia, Emilia Romagna, Sardinia	Carlucci *et al*., 2015 Dissanayake *et al*., 2016b, 2017 Luchi *et al*., 2014 Manca *et al*., 2020
syn. *Do. italica*	*Cupressus* sp. *Ligustrum* sp. *Melia azedarach* *Prunus* sp. *Rosa canina* *Rubus* sp.	Cankers	Emilia Romagna, Umbria	Dissanayake *et al.,* 2017

(Continued)

Table 1. (Continued).

Species	Host	Symptoms	Region	Reference
Do. sempervirentis	*Cytisus* sp. *Fraxinus excelsior*	Cankers, dieback	Umbria, Veneto	Dissanayake *et al.*, 2016b, 2017 Linaldeddu *et al.*, 2020b
Do. symphoricarpicola	*Cornus sanguinea* *Corylus avellane* *Laburnum alpinum* *L. anagyroides* *Laurus nobilis* *Symphoricarpos* sp. *Sambucus nigra*	Cankers and branch dieback	Emilia Romagna, Sardinia	Dissanayake *et al.,* 2016b Li *et al.*, 2014 Linaldeddu *et al.*, 2016b
Do. vidmadera	*Fraxinus ornus*	Dead branch	Emilia Romagna	Dissanayake *et al.,* 2016b
Do. viticola	*Citrus* sp. *Morus* sp.	Dieback	Sicily, Emilia Romagna	Bezerra *et al.*, 2021 Rathnayaka *et al.*, 2022
Eutiarosporella dactylidis	*Arrhenatherum elatius* *Avenella flexuosa* *Dactylis glomerata*	Stem cankers	Emilia Romagna	Dissanayake *et al.*, 2016b Wijesinghe *et al.*, 2021
Lasiodiplodia citricola	*Acacia dealbata* *Acacia retinoides* *Persea americana* *Vitis vinifera*	Cankers, wood necrosis, V-shaped necrotic sectors	Apulia, Molise, Sicily	Carlucci *et al.*, 2015 Costanzo *et al.,* 2022 Fiorenza *et al.*, 2023 Raimondo *et al.*, 2019
L. hormozganensis	*Quercus cerris*	-	-	Kee *et al.*, 2019
L. iraniensis	*Vitis vinifera*	-	-	Jayawardena *et al.*, 2018
L. laeliocattleyae	*Laeliocattleya* sp.	-	-	Custodio *et al.*, 2018 Dissanayake *et al.,* 2016b Kee *et al.*, 2019
L. mediterranea	*Quercus ilex* *Vitis vinifera*	Canker with V-shaped necrotic sectors and dieback	Sardinia	Linaldeddu *et al.*, 2015a
L. theobromae	*Mangifera indica* *Olea europaea* *Persea americana* *Rosa canina* *Vitis vinifera*	Cankers, shoot blight, wood necrosis, V-shaped necrotic sectors	Apulia, Emilia Romagna, Molise, Piedmont, Sicily	Aiello *et al.*, 2022 Bertetti *et al.*, 2013 Burruano *et al.*, 2008 Carlucci *et al.*, 2013, 2015 Mondello *et al.*, 2013 Raimondo *et al.*, 2019 Wijayawardene *et al.*, 2016 Wijesinghe *et al.*, 2021
Macrophomina phaseolina	*Beta vulgaris* *Cicer arietinum* *Citrullus* sp. *Cucumis melo* *Fragaria* × *ananassa* *Glycine max* *Helianthus annuus* *Hibiscus* sp. *Opuntia humifusa* *Osteospermum* sp. *Persea americana* *Phaseolus vulgaris* *Prunus persica* *Solanum tuberosum*	Dry root rot, collar rot, charcoal rot and soft stem rot, dark brown discoloration	Basilicata, Calabria Campania, Sardinia	Dell'Olmo *et al.*, 2022 Faedda *et al.,* 2016 Fiorenza *et al.*, 2023 Gerin *et al.*, 2018 Infantino *et al.*, 2021 Poudel *et al.*, 2021
Mucoharknessia anthoxanthi	*Anthoxanthum odoratum*	-	Emilia Romagna	Dissanayake *et al.,* 2016b

(Continued)

Table 1. (Continued).

Species	Host	Symptoms	Region	Reference
Neofusicoccum australe	*Eucalyptus camaldulensis* *Mangifera indica* *Myrtus communis* *Olea europaea* *Pinus nigra* *Vitis vinifera*	Leaf chlorosis, crown thinning, shoot and branch dieback, sunken Cankers, epicormic shoots gummosis, V-shaped necrotic sectors, fruit rot	Apulia, Sardinia, Sicily	Deidda *et al*., 2016 Ismail *et al.,* 2013 Lazzizera *et al*., 2008b Linaldeddu *et al*., 2010b, 2015a Luchi *et al*., 2014 Nicoletti *et al*., 2014
N. batangarum	*Opuntia ficus-indica*	Cankers	Sicily	Aloi *et al*., 2020 Masi *et al*., 2020b Santagata *et al.,* 2022
N. buxi	*Buxus sempervirens*	Leaf spots	Liguria	Cecchi *et al*., 2020
N. cordaticola	*Vitis vinifera*	-	-	Jayawardena *et al*., 2018 Sakalidis *et al*., 2013
N. cryptoaustrale	*Olea europaea* *Pistacia lentiscus* *Persea americana* *Vitis vinifera*	Cankers, dieback, V-shaped necrotic sectors	Sardinia	Fiorenza *et al*., 2023 Linaldeddu *et al*., 2015a, 2016c Yang *et al*., 2017
N. hellenicum	*Pistacia vera*	Shoot and panicle blight	Sicily	Gusella *et al*., 2022
N. luteum	*Cinnamomum camphora* *E. camaldulensis* *Erica arborea* *Olea europaea* *Persea americana* *Pinus pinea* *Pistacia vera** *Pistacia lentiscus* *Viburnum* sp. *Vitis vinifera*	Leaf chlorosis, crown thinning, shoot and branch and twig dieback, sunken cankers, epicormic shoots gummosis, V -shaped necrotic sectors, fruit rot	Apulia, Liguria, Sardinia, Sicily	Carlucci *et al.,* 2013 Deidda *et al*., 2016 Fiorenza *et al*., 2023 Gusella *et al*., 2023a Linaldeddu *et al*., 2015b, 2016c Luchi *et al*., 2014 Zhang *et al*., 2020
N. mediterraneum	*Arbutus unedo** *E. camaldulensis* *Ficus microcarpa* *Juglans regia* *Olea europaea* *Pistacia vera*	Leaf chlorosis, crown thinning, shoot and branch dieback, sunken cankers, epicormic shoots gummosis, V-shaped necrotic sectors	Apulia, Lazio, Sardinia*, Sicily	Brunetti *et al*., 2022 Deidda *et al*., 2016 Fiorenza *et al*., 2022 Gusella *et al.,* 2020b, 2022 Manetti *et al.,* 2023 This study*
N. occulatum	*Platanus hybrida*	-	-	Yang *et al*., 2017

(Continued)

Table 1. (Continued).

Species	Host	Symptoms	Region	Reference
N. parvum	*Acer pseudoplatanus* *Acacia melanoxylon* *Brachychiton* spp. *Cannabis sativa* *Castanea sativa* *Citrus × limon* *Citrus* spp. *Cinnamomum camphora* *Corylus avellana* *Eriobotrya japonica* *E. camaldulensis* *Eupatorium cannabinum* *Ficus carica* *Ficus microcarpa* *Fraxinus excelsior* *Juglans regia* *Malus* sp. *Mangifera indica* *Meryta denhamii* *Microcitrus australasica* *Olea europaea* *Olea oleaster* *Persea americana* *Pinus pinea* *Punica granatum* *Rhododendron* sp. *Quercus ilex* *Quercus robur* *Quercus suber* *Raphiolepis indica* *Rhododendron* sp. *Rubus fruticosus* *Salix* sp. *Torilis arvensis* *Ulmus hollandica* *Vaccinium* sp. *Vitis vinifera*	Cankers, wedge-shaped necrotic sectors, chlorosis, leaf and shoot blight, leaf drop, fruit rot, gummosis, V-shaped necrotic sectors, twig dieback wilting shoots,	Abruzzo, Apulia, Basilicata, Emilia Romagna, Friuli Venezia Giulia, Lazio, Lombardy, Molise, Piedmont, Sardinia, Sicily, Tuscany, Veneto	Aiello *et al*., 2020, 2022 Alberti *et al.,* 2018 Aloi *et al*., 2021 Bezerra *et al*., 2021 Carlucci *et al*., 2013, 2015 Dardani *et al*., 2023 Deidda *et al*., 2016 Dissanayake *et al*., 2017 Faedda *et al*., 2018 Fiorenza *et al*., 2022 Garibaldi *et al*., 2011 Giambra *et al*., 2016 Guarnaccia *et al*., 2016, 2020a Gusella *et al*., 2020a, 2020b, 2021, 2023a, 2023b Ismail *et al.,* 2013 Linaldeddu *et al*., 2007, 2014, 2015a, 2020b Luchi *et al*., 2014 Manca *et al*., 2020 Mang *et al.,* 2022 Mondello *et al*., 2013 Moricca *et al*., 2012 Polizzi *et al*., 2023 Raimondo *et al*., 2019 Riccioni *et al*., 2017 Seddaiu *et al*., 2021 Sidoti, 2016 Spagnolo *et al*., 2011 Waqas *et al*., 2022 Wijesinghe *et al*., 2021 Zlatkovic *et al*., 2019
syn. *N. italicum*	*Vitis vinifera*	-	-	Marin-Felix *et al*., 2017
N. stellenboschiana	*Olea europaea*	Cankers, branch and twig dieback	Apulia	Manetti *et al*., 2023
N. vitifusiforme	*Eriobotrya japonica* *E. camaldulensis* *Mangifera indica* *Olea europaea* *Pinus nigra* *Vitis vinifera*	Leaf chlorosis, crown thinning, shoot and branch dieback, sunken cankers, epicormic shoots gummosis, V-shaped necrotic sectors	Sardinia, Sicily	Deidda *et al*., 2016 Dissanayake *et al*., 2016b Giambra *et al*., 2016 Luchi *et al*., 2014 Mondello *et al*., 2013 Moral *et al*., 2010 Zhang *et al*., 2020
Neoscytalidium dimidiatum	*Citrus sinensis* *Meryta denhamii*	Shoot blight, canker, gummosis, dieback	Sicily	Gusella *et al*., 2023b Polizzi *et al.,* 2009
Sardiniella celtidis	*Celtis australis*		Emilia Romagna	Hyde *et al*., 2017
S. urbana	*Celtis australis*	Shoot and branch dieback, sunken cankers	Sardinia	Linaldeddu *et al.,* 2016a

* New host-pathogen interactions reported in this study.

al., 2020, 2022; Gusella *et al.,* 2020a, 2020b, 2021, 2022; Linaldeddu *et al.,* 2020a; Bezerra *et al.,* 2021; Fiorenza *et al.,* 2022, 2023), and these infections are often caused by multiple pathogen genera that may play different roles in infection processes of host plants.

Botryosphaeriaceae have been commonly recorded in agro-ecosystems, and in nurseries, urban landscapes and forest ecosystems including timber plantations. The plant propagation processes in nurseries are crucial for many production sectors (ornamentals, forestry and fruit crops). Fungal latency, in conjunction with intercontinental plant transport without adequate quarantine, can lead pathogen spread, which is why preventive detection and adequate control strategies are always needed to limit the destructive potential of *Botryosphaeriaceae*. As summarized in Figure 1, *Botryosphaeriaceae* inoculum (spores or mycelium) can be present during initial propagation steps in nurseries, which is why symptoms can occur and plant material is discarded before being sale, but the pathogens can also remain latent. From nurseries, infected plant material can be shipped around the world, and symptoms can appear months or years later once the plants are transplanted in the field. For this reason, careful hygiene during propagation and healthy plant material are crucial for avoiding infection establishment in nurseries. Before symptom appearance during propagation steps or in orchards, diagnostic analyses could identify latent *Botryosphaeriaceae* in the plant tissues. Traditional laboratory analyses, such as isolation on growth media, are still valuable for determining frequency of active fungal population within plant tissues. These traditional methods are usually time-consuming compared to molecular diagnostic methods. Real-time PCR has been demonstrated to be an important tool for detecting latent infections and to investigate canker pathogen epidemiology (Luo *et al*., 2017, 2019, 2020; Romero-Cuadrado *et al*., 2023). Once *Botryosphaeriaceae* become established in an orchard, transmission of inoculum (mycelium, pycnidia or perithecia overwintering in old cankers, fruit mummies or within the buds) (Michailides, 1991) can occur through human activities (e.g., pruning, irrigation), and animals such as birds and insects (Michailides and Morgan, 2016), and environmental factors such as rain and wind. Riparian vegetation near the orchards can also be important (Figure 1), as many wild species, bushes and forest trees can be important dissemination pathways for *Botryosphaeriaceae* (Ma *et al*., 2001).

Forest ecosystems and timber plantations

Forest ecosystems (natural, seminatural and artificial) cover 36% of the Italian territory, and are important for human services and income (Ferrara *et al.,* 2017; Agnoletti *et al.,* 2022). The Italian forest heritage includes a wide variety of ecosystems, spanning from natural Mediterranean evergreen sclerophyllous formations to Norway spruce plantations in the Alps (Gasparini *et al.,* 2022). The health status of these ecosystems is continuously threatened by several native and exotic pathogens, including species of *Botryosphaeriaceae* (Santini *et al.,* 2013; Luchi *et al.,* 2014; Moricca *et al*., 2016; Linaldeddu *et al*., 2020b). A meta-analysis of the literature has allowed determination of the occurrence of 37 *Botryosphaeriaceae* species and 129 host-pathogen interactions in natural ecosystems and timber plantations. This analysis showed some distribution patterns partially explained by the host preference of some species: *D. sapinea* for *Pinus* spp., *D. corticola* for *Quercus* spp., *D. cupressi* for *Cupressus* spp. and *S. urbana* for *Celtis australis*. In contrast, many species, especially those that are polyphagous, have irregular geographic distributions (Linaldeddu *et al.,* 2014, 2016a, 2020b; Luchi *et al.,* 2014; Batista *et al.,* 2021).

Different species of *Diplodia* and *Neofusicoccum* are increasing threats to forest ecosystems in Italy (Linaldeddu *et al.,* 2011a, 2014, 2020b; Deidda *et al.,* 2016; Manca *et al.,* 2020). In particular, *D. corticola, D. fraxini, D. insularis, D. scrobiculata, D. subglobosa, N. australe, N, luteum, N. mediterraneum* and *N. parvum* are associated with disease symptoms including leaf spot, fruit rot, shoot blight, branch dieback, sunken canker, decline and mortality on different shrubs and forest trees (Figure 2). Since 2010, an unusual decline and mortality of young and mature *Eucalyptus camaldulensis* trees has been observed in several plantations in Sardinia (Deidda *et al.,* 2016). Five species of *Neofusicoccum,* namely *N. australe*, *N. luteum*, *N. mediterraneum*, *N. parvum* and *N. vitifusiforme*, were consistently isolated from trees showing symptoms of leaf chlorosis, shoot and branch dieback, sunken cankers, epicormic shoots and exudations of kino gum. In particular, *N. australe* was the most frequently isolated fungus, and other studies conducted in grapevines, almond and olive orchards adjacent to eucalypt plantations showed that this species was isolate from sunken cankers and fruit rots, demonstrating its invasive potential in the Mediterranean region (Linaldeddu, personal communication).

Among the many symptoms caused by *Botryosphaeriaceae* on woody hosts, wedge-shaped necrotic sectors visible in stem cross section associated with the sunken cankers are frequent and typical of this group of pathogens (Figure 3).

Although several species of *Botryosphaeriaceae* have been described from different forest ecosystems and Ital-

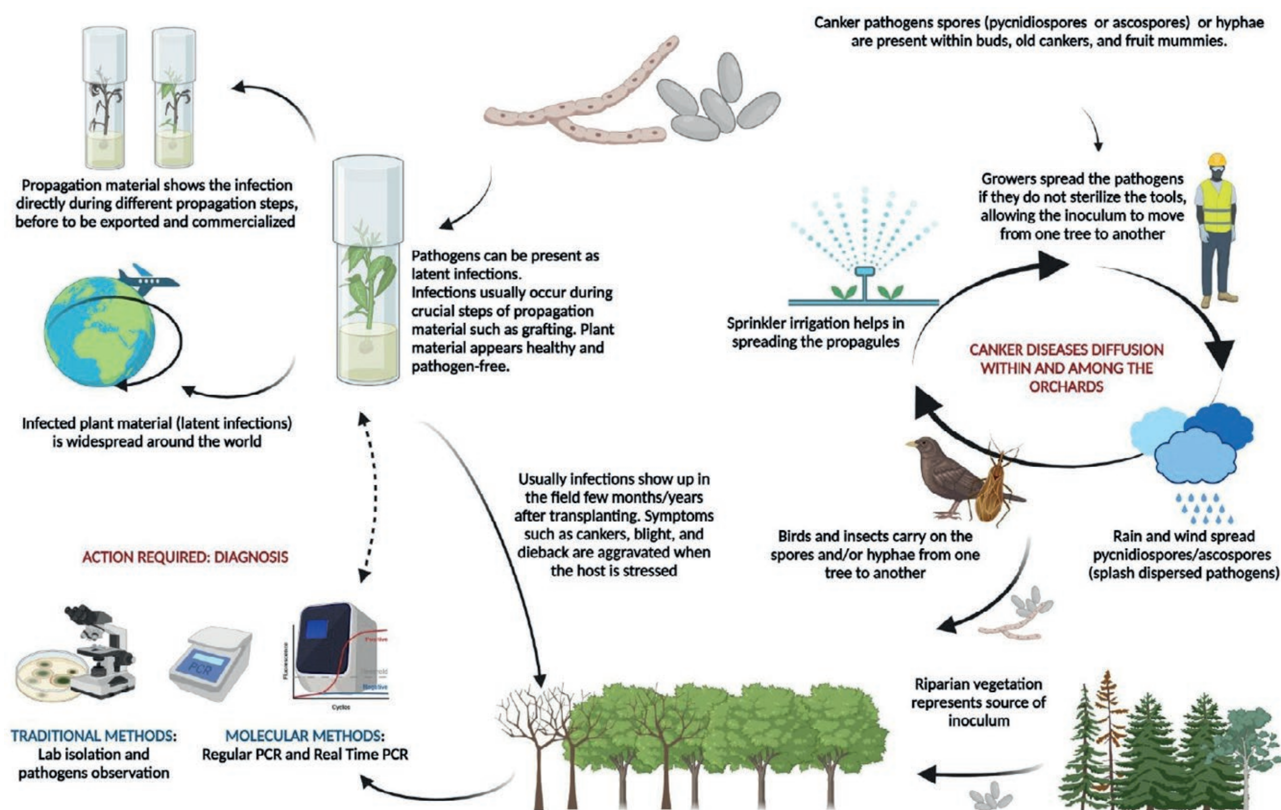

Figure 1. Cycle of *Botryosphaeriaceae* dispersion from the nurseries to the field (created with BioRender).

ian regions, the diversity and distribution of this group of fungi has not been widely of the 129 host-pathogen interactions known, only 35 were verified by pathogenicity tests under experimental conditions. Given the small number of sampling efforts in some regions, and current climate changes which may favour the invasiveness of some of these pathogens, further studies should target these invasive fungi to develop the basis for suitable disease management strategies.

Ornamental and urban plants

Proliferation of phytopathological cases ascribable to *Botryosphaeriaceae*, especially in nurseries, has occurred where infections start latently and continue once plants are transplanted in open fields (Figure 4). Most of the symptomatic and/or dead plants that have been observed in the field were probably already infected (asymptomatic) in nurseries. *Botryosphaeriaceae* infections occur easily during some propagation steps, including grafting, has been observed with *Acacia* spp. infected by *L. citricola* (Figure 4 h) (Costanzo *et al.*, 2022). Among the *Botryosphaeriaceae* spp., *N. parvum* has been consistently isolated from different hosts. Severe infections causing trunk cankers, massive gummoses and canopy dieback were observed in nurseries of the ornamental *Brachychiton* spp. (Figure 4 e) (Gusella *et al.*, 2021), as well as wood necroses and dieback on ornamental fig (*Ficus carica*) cuttings (Figure 4 f) (Aiello *et al.*, 2020), and on Indian hawthorn (*Rhaphiolepis indica*) (Figure 4 g) (Gusella *et al.*, 2020a).

As mentioned above, ornamental crops in urban environments are important sources of *Botryosphaeriaceae* inoculum. Urban trees often grow in non-native environments, and *Botryosphaeriaceae*, being endophytes, occupy the endophytic niche left open, normally occupied in native habitats, by horizontally acquired endophytes (Slippers and Wingfield, 2007). Surveys conducted in 2019 and 2020 on *Ficus microcarpa* on several tree-lined streets, squares and public parks in Catania and Siracusa provinces (Sicily, southern Italy) revealed common presence of shoot and branch canker, canopy defoliation, internal wood necroses and dieback (Figure 4, a to d). Multi-locus phylogenetic analyses showed that *B. dothidea*, *N. mediterraneum*, and *N. parvum* were responsible for the tree decline (Fiorenza *et al.*, 2021).

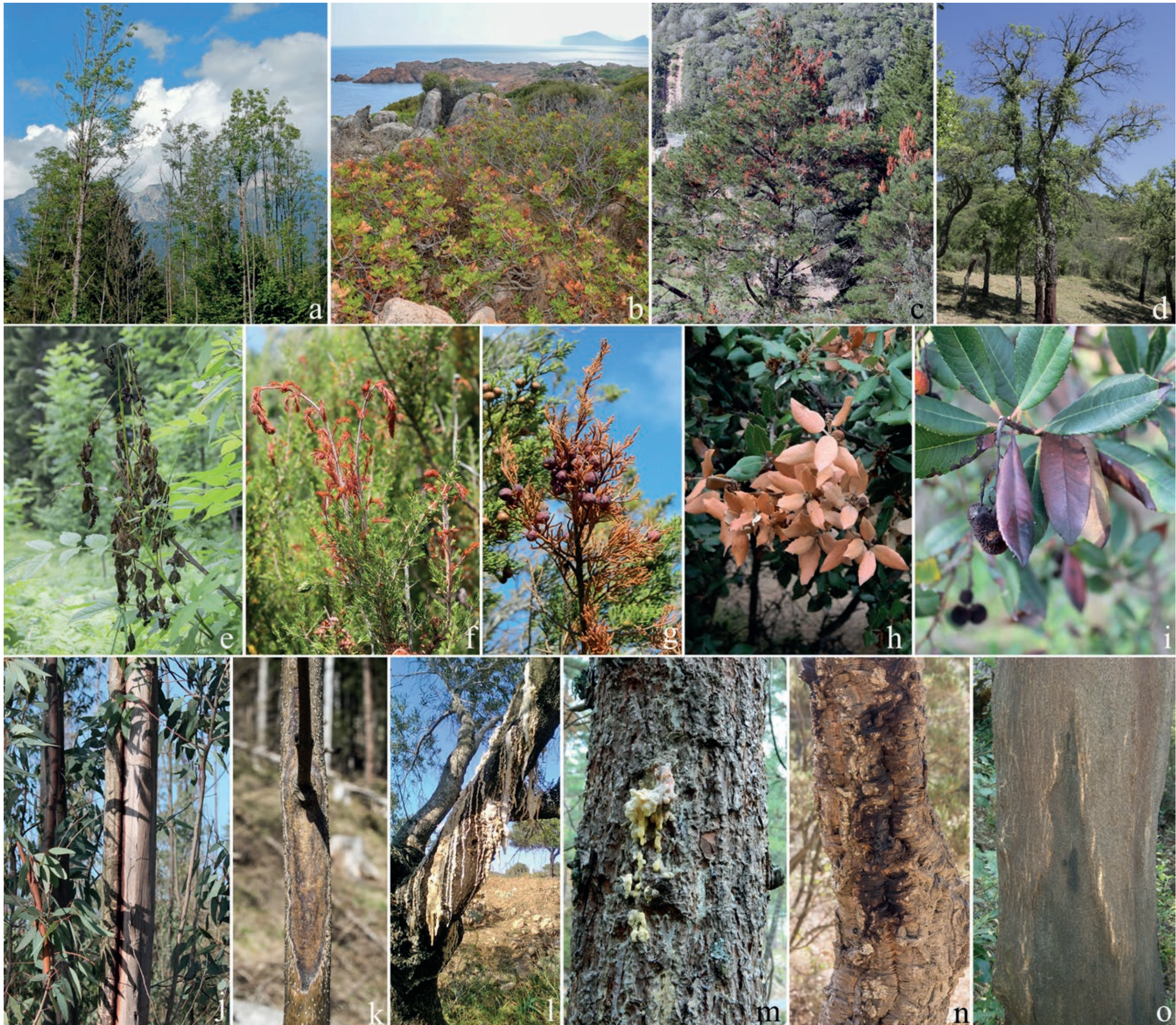

Figure 2. Overview of external disease symptoms caused by *Botryosphaeriaceae* on different forest trees and shrubs. (a) progressive canopy dieback caused by *Diplodia fraxini* on *Fraxinus excelsior*; (b) *Diplodia insularis* on *Pistacia lentiscus*; (c) *Diplodia sapinea* on *Pinus radiata*; (d) *Diplodia corticola* on *Quercus suber*; (e) shoot blight caused by *D. fraxini* on *F. excelsior*; (f) *Neofusicoccum luteum* on *Erica arborea*; (g) *Diplodia africana* on *Juniperus phoenicea*; (h) *D. corticola* on *Quercus ilex*; (i) *Neofusicoccum mediterraneum* on *Arbutus unedo*; (j) sunken canker with exudations caused *by Neofusicoccum australe* on *Eucalyptus camaldulensis*; (k) *D. fraxini* on *F. excelsior*; (l) *Neofusicoccum parvum* on *Olea oleaster*; (m) *D. sapinea* on *Pinus radiata*; (n and o) *D. corticola* on *Q. suber*.

Branch cankers and dieback caused by *N. parvum* and *Neoscytalidium dimidiatum* were also observed on *Meryta denhamii* in a historical botanical garden (Gusella *et al*., 2023b).

Tropical crops

In recent years, increased occurrence of symptoms caused by *Botryosphaeriaceae* has been observed on mango and avocado plants in Sicily (Southern Italy) (Figures 5 to 7). Cultivation of these plants started in the Catania province (eastern Sicily) in 1980. Thereafter, mango and avocado cultivation expanded to the other provinces of Sicily, and to Calabria and Apulia. These tropical crops are alternatives o citrus, and they are economically important in European markets. Botryosphaeriaceae infections may occur pre- and post-harvest, and these compromise plant growth and/or fruit quality

Figure 3. Overview of internal disease symptoms caused by *Botryosphaeriaceae* on different forest trees and shrubs: (a) cross section of branches showing the characteristic wedge-shaped necrotic sector caused by *Neofusicoccum mediterraneum* on *Arbutus unedo*; (b) *Neofusicoccum parvum* on *Olea oleaster*; (c) *Diplodia insularis* on *Pistacia lentiscus*; (d) *Dothiorella* sp. on *Rhamnus alaternus*; (e) *Sardiniella urbana* on *Celtis australis*; (f) *Diplodia cupressi* on *Cupressus sempervirens*; (g) *Diplodia seriata* on *Corylus avellana*; (h) *Neofusicoccum australe* on *Eucalyptus camaldulensis*; (i) *Neofusicoccum parvum* on *Fagus sylvatica*; (j) *Diplodia fraxini* on *Fraxinus excelsior*; (k) *Diplodia sapinea* on *Pinus mugo*; (l) *Botryosphaeria dothidea* on *Rhododendron ferrugineum*; (m) *Diplodia corticola* on *Quercus ilex*, (n) *Q. pubescens*, (o) *Q. robur* and (p) *Q. suber*.

leading to substantial yield losses and decreases in market value. After the first report of dieback caused by *N. parvum* on mango (Ismail *et al.*, 2013), surveys carried out between 2014 and 2019 detected severe symptoms of woody canker, shoot blight, and dieback on different cultivars of young mango plants (Kent, Keitt, Sensation,

Figure 4. Disease symptoms caused by *Botryosphaeriaceae* on ornamental plants in urban environments and nurseries: (a and b) diseased plant of *Ficus microcarpa* with defoliation and shoot dieback all over the canopy; (c and d) bark discolored and cracked along the branch and internal tissues showing blackish V-shape lesion of *F. microcarpa*; (e) canker and gummosis of *Brachychiton* sp.; (f) internal discoloration of *Ficus carica* cutting; (g) necrosis at the bottom of the leaves of *Rhaphiolepis indica*, moving from petioles upward through the mid rib and blade; (h) canker at the graft union on young *Acacia dealbata* plant grafted on *A. retinodes*.

Osteen, and Kensington Pride) in north-eastern Sicily (Figure 5, a to e). Morphological and molecular characters and pathogenicity tests identified the associated pathogens, including *B. dothidea*, *L. theobromae* and *N. parvum* (Aiello *et al.,* 2022). Among these, *N. parvum* was widespread on tropical crops in the area (Guarnaccia *et al.,* 2016; Aiello *et al.,* 2022), and on citrus (Bezerra *et al.,* 2021). This species was also detected causing seedling blight of mango (*Mangifera indica*) in a nursery (Polizzi *et al.,* 2023). Mango fruit symptoms were not reported in Italy, but different authors showed the severe rot symptoms caused by *Botryosphaeriaceae* on the stem ends of fruit when infections commenced from pedicles or from fruit surfaces (Figure 7, d to f) (Ni *et al*., 2010). Further and future investigations in Italy will aim to assess the spread of symptoms on mango fruit, which is becoming a crop of increasing relevance.

In 2016, surveys on avocado orchards showed branch canker and fruit stem-end rot, caused by *N. parvum* in association with other *Diaporthaceae* and *Glomerellaceae* (Guarnaccia *et al.,* 2016). The same *Neofusicoccum parvum* was also reported, together with *N. stellenboschiana*, causing branch canker on avocado in Greece (Guarnaccia *et al*., 2020b). Studies of avocado diseases in Sicily continued, and during 2020/2021, surveys were conducted in Sicily on 11 orchards, to investigate the etiology of branch canker and dieback (Figure 6, a to e). Among these orchards, four showed constant presence

Figure 5. Disease symptoms caused by *Botryosphaeriaceae* on field-grown mango plants: (a) shoot dieback; (b and c) external and internal canker; (d) internal necrotic tissue; (e) external canker and bark cracking.

of *Botryosphaeriaceae*. Phylogenetic analyses identified five *Botryosphaeriaceae* species: *B. dothidea*, *L. citricola*, *M. phaseolina*, *N. cryptoaustrale* and *N. luteum*. The symptoms included cankers on shoots and branches, and trunk and shoot dieback. Cankers were usually associated with reddish sap that became white/beige with age. Bark was cracked, darkly discoloured and sometimes slightly sunken. Occasionally, white sugar-like powder was present on the bark surface. Under the bark, canker wood tissues were reddish or light brown to black, and variable in shape. Characteristic wedge-shaped discolouration affecting the xylem was visible in cross sections. Under high disease pressure, wilting of shoots and leaves was also observed (Fiorenza *et al.*, 2023).

On fruit, external symptoms developed as dark brown to black rot sometimes affected the stem ends or most of the fruit epicarps. Internally, the flesh had discoloured vascular streaking (Figure 7, a to c). As the fruit ripened, the rots progressed and resulted in general discolouration, brown flesh and fruit shriveling. Occasionally, signs of the fungus (mycelium and/or fruiting bodies) were observed on symptomatic tissues.

Fruit trees

Pistachio

In Sicily, investigations of agricultural and ornamental crops showed presence of *Botryosphaeriaceae*-caused diseases on pistachio that had not been previously recorded (Polizzi, personal communication). In 2019 field investigations of pistachio orchards showed the presence of *Botryosphaeriaceae* pathogens on these plants. Pistachio (*Pistacia vera*) is historically important for the Sicily economy (Barone and Marra, 2004), and is

Figure 6. Disease symptoms caused by *Botryosphaeriaceae* on field-grown avocado plants: (a) severe shoot dieback in the canopy; (b) branch dieback; (c) infection starting from pruned wound; (d and e) external canker and discoloured internal tissues.

traditionally linked to the territory of Bronte (Catania province) where natural plantings of pistachio are present. These orchards are defined as "natural pistachio plantings" obtained by grafting *in situ* spontaneous terebinth plants (*Pistacia terebinthus*) that grow widespread in the volcanic mountain soils of the area (Barone *et al.*, 1985). More recent pistachio orchards in Agrigento and Caltanissetta provinces, named "new" orchards characterized by rational design, scheduled irrigation and fertilization, and mechanical harvest, are increasing in the territory (Marino and Marra, 2019).

In these new orchards, symptomatic fruit panicles, leaves and shoots were observed during summer of 2019. Fruit showed black rounded spots on the epicarps enlarging with time (also including rachis black discoloration) (Figure 7 g). Leaves were necrotized and shoots showed dieback, wood discolouration (i.e., necrotic, and sunken lesions on lignified tissues), and external cankers (Gusella *et al.*, 2022). Morphological and phylogenetic analyses showed the presence of *B. dothidea*, *N. hellenicum* and *N. mediterraneum* causing Botryosphaeria panicle and shoot blight in Italy, with *N. mediterraneum* the most prevalent species affecting pistachio in Sicily (Gusella *et al.*, 2022). In countries where pistachio cultivation is intensive (e.g. the United States of America), this disease has been well-known since the early 1990's, when the causal agent was identified as *B. dothidea* (Michailides, 1991). Later, with progress in multi-locus phylogenetic analyses, more than one pathogenic species was shown to be involved, so the condition is better defined as a disease complex (Moral *et al.*, 2010; Chen *et al.*, 2014). In Italy, before the investigations of 2019, a report of 'Botryosphaeria' dieback on pistachio in 1938 attributed the disease to *Botryodiplodia pistaciae* (Cristinzio, 1938). In Sicilian nut crops, *B. dothidea*, *N. mediterraneum* and *N. parvum* were also reported on English walnut (*Juglans regia*), causing shoot and trunk canker, shoot blight, and necroses (Gusella *et al.*, 2020b).

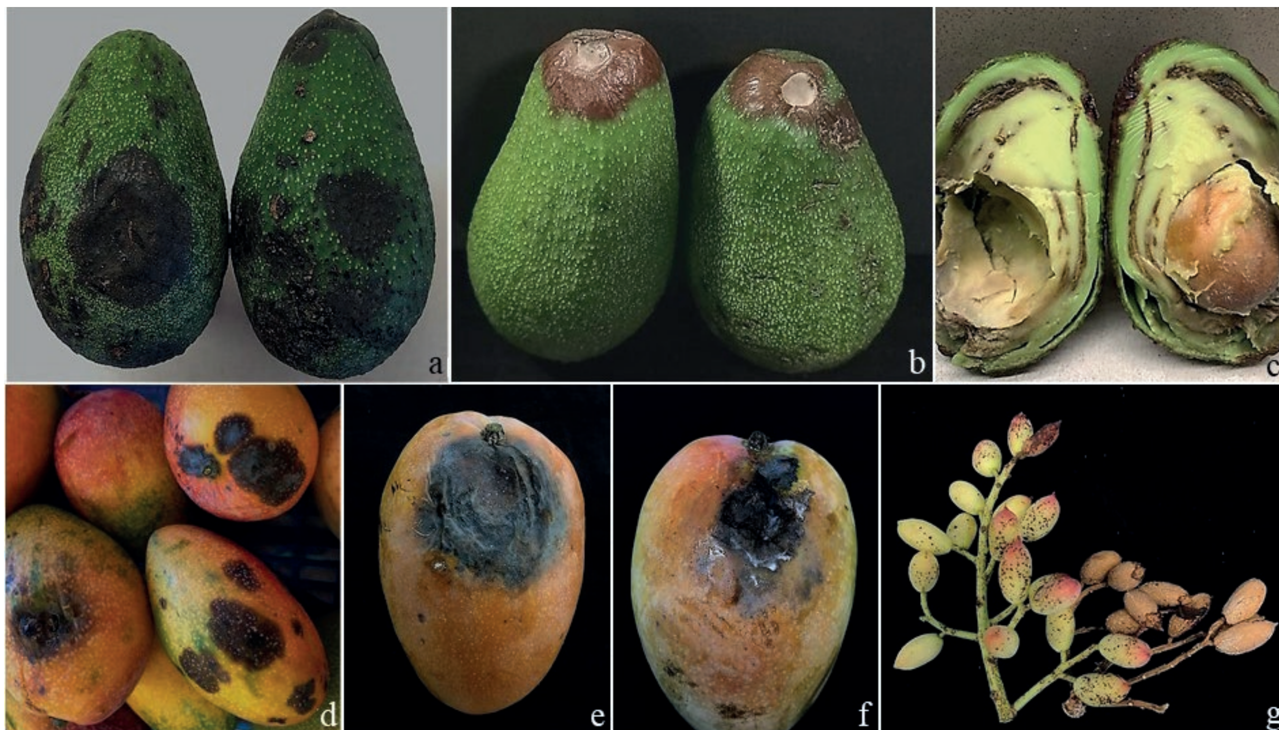

Figure 7. Disease symptoms caused by *Botryosphaeriaceae* on fruit: (a and b) fruit rot affected most of the epicarps and the stem-ends of avocado fruit; (c) discoloured vascular streaking of avocado flesh; (d) rot of mango fruit; (e and f) stem-end rot of mango fruit; (g) fruit spot and panicle blight of pistachio.

Grapevine

Investigations of grapevine wood diseases in Italy during the last 15 years have shown conspicuous presence of *Botryosphaeriaceae* species as causal agents of severe diseases. To date, 16 species in *Botryosphaeriaceae* have been reported and described as important vineyard pathogens. The main reports assessed presence of these fungi in Apulia, Sardinia and Sicily, the greatest Italian production regions for wine and table grapes. The grapevine symptoms caused by *Botryosphaeriaceae* species infections are of two kinds; external and internal symptoms. External symptoms include leaf wilting, fruit rots, bud necroses and perennial cankers, cordon dieback, and plant decline (Figure 8 a). Internal symptoms include wedge-shaped necroses in stem cross sections, and brown streaking below the bark in longitudinal sections (Figure 8, b and c). The first Italian tentative associations of bark cankers, dieback, and leaf chloroses with *Botryosphaeriaceae* species were reported by Cristinzio (1979), and Rovesti and Montermini (1987), who associated *D. seriata* with grapevine dieback. Burruano *et al*. (2008) and Carlucci *et al*. (2009) provided the first reports of cankers of grapevines caused by a *Botryosphaeriaceae* species in, respectively, Sicily and in Apulia regions. In Sicily, *Lasiodiplodia theobromae* was, for the first time, considered responsible for wood cankers and mild leaf chloroses, while in Apulia, nine species (*B. dothidea*, *D. corticola*, *D. mutila*, *D. seriata*, *Do. iberica*, *Do. sarmentorum*, *N. luteum*, *N. parvum* and *L. theobromae*) were found to be responsible for grapevine dieback, often in association with Esca complex symptoms. In particular, *Do. sarmentorum* and *D. corticola* were isolated for the first time from wood streaking on grapevines by Carlucci *et al*. (2009) and Carlucci and Frisullo, (2009). Spagnolo et al., 2011 isolated *N. parvum*, *D. dothidea* and *D. seriata* in Tuscany, while Mondello *et al*. (2013) reported *N. vitifusiforme* for the first time, on grapevines in western and central Sicily, and described decline symptoms similar to those observed by Burruano *et al*. (2008). Linaldeddu *et al*. (2015 a) isolated, and associated *D. africana* and *D. olivarum* with grapevines, for the first time in Sardinia and elsewhere in the world. These authors also isolated and described *L. exigua* and *L. mediterranea* as new from grapevines. Carlucci *et al*. (2015) first described the presence of *L. citricola* associated with grapevine wood cankers and dieback. That paper described symptoms on host samples collected

during 2012/2013 surveys, and the symptoms detected during pathogenicity tests carried out on green shoots and 1-year-old canes of two cultivars (‘Lambrusco’ and ‘Sangiovese’) with the nine species of *Botryosphaeriaceae* listed above. The study demonstrated that different species produced different severities, and that all the species caused wood discolourations, confirming the fungi as primary causes of dieback and decline of vineyards in southern Italy. Although *Botryosphaeriaceae* fungi have been demonstrated to be severe pathogens for grapevines, they have always been isolated together with other fungal pathogens known to be responsible for grapevine trunk diseases (GTDs), such as those associated with the Esca disease complex (Carlucci *et al*., 2015; Raimondo *et al*., 2019) and black foot (Carlucci *et al*., 2017).

Olive

The presence of *Botryosphaeriaceae* fungi associated with decline of olives in Italy is not well defined, although several studies on olive diseases have reported and described the involvement of some of these fungi. Lazzizera *et al*., (2008a, 2008b), during surveys carried out in southern Italy (Apulia and Basilicata regions), isolated many fungi belonging to *Botryosphaeriaceae*, from rotted olive drupes. These fungi included the new species *D. olivarum*. They also isolated, from rotted drupes, *D. seriata, D. pinea, D. scrobiculata, B. dothidea, N. australe, N. vitifusiforme, N. mediterraneum* and *N. parvum*. They reported that the most aggressive pathogens on olive drupes, among those tested, were *N. australe, N. vitifusiforme* and *D. olivarum*. Carlucci *et al.* (2013) showed that some of the above-mentioned fungi caused severe symptoms in olive wood tissues, and were responsible for reduced olive yields in Apulia region. These studies associated *B. dothidea, D. mutila, D. seriata, L. theobromae, N. luteum* and *N. parvum* with severe damage to wood tissues, although other fungal pathogens, including *Phaeoacremonium* spp. and *Pleurostoma richardsiae* were also severe olive pathogens. Carlucci *et al.* (2020) showed that the olive quick decline syndrome (OQDS) that occurs in southern Italy (Lecce province, Apulia region) is due mainly to *Xylella fastidiosa*, but has also been associated with several lignicolous fungi including *Botryosphaeriaceae*, such as *B. dothidea, D. seriata, N. luteum, N. parvum* and *N. mediterraneum*. In particular, *N. mediterraneum* was reported by Brunetti *et al.* (2022) as one of the most aggressive fungal pathogens involved in OQDS, and caused olive twig dieback in Apulia region. Their data, supported by pathogenicity tests, agree with earlier studies (Carlucci *et al*., 2020). The symptoms observed on olive trees consisted of wood discolourations in stem cross and longitudinal sections, cankers, dieback and general decline, often associated with leaf yellowing, wilting and/or leaf scorch (Figure 8, d to g) (Carlucci, personal communication).

Citrus

Citrus cultivation is globally important. In Europe, Greece, Italy, Portugal, and Spain are important citrus producers (FAOSTAT, 2019). Several abiotic and biotic factors are involved in rot and gummosis of citrus trunks and primary branches. Frost damage, sun scald, or irregular water distribution affect infection by *Ascomycetes* and *Basidiomycetes* (Timmer *et al*., 2000). Several trunk pathogens are known to cause diseases of citrus in Europe (Guarnaccia and Crous, 2017; Sandoval *et al*., 2018; Leonardi *et al*., 2023), and focus has been given to *Botryosphaeriaceae*. Several species of *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, and *Neoscytalidium* have been documented as affecting citrus hosts (Figure 8 h). For example, *Ne. dimidiatum* has been identified as the cause of citrus branch canker in California (Mayorquin *et al*., 2016) and Italy (Polizzi *et al*., 2009). Bezerra *et al.* (2021) demonstrated occurrence, genetic diversity, and pathogenicity of *Botryosphaeriaceae* associated with symptomatic *Citrus* spp. in Greece, Italy, Portugal, Malta and Spain. Extensive sampling was carried out, along with morphological and DNA phylogenetic analyses of potential isolated pathogens. Symptomatic plants were observed in all the investigated citrus orchards and regions, and all isolates used in pathogenicity tests caused lesions on the wood of inoculated citrus plants. Phylogenetic analyses identified four *Diplodia* species, with *D. pseudoseriata* being the most prevalent, followed by *D. seriata*, *D. olivarum*, and *D. mutila*. The only *Neofusicoccum* spp. identified were *N. parvum*, *N. luteum*, and *N. mediterraneum*. Additionally, *Do. viticola* and *L. theobromae* were also recorded, and *Diplodia* and *Neofusicoccum* spp. were the dominant genera reported. Among the inoculated species, *D. seriata*, *D. olivarum*, *L. theobromae*, *N. mediterraneum*, *N. luteum*, and *N. parvum* were highly aggressive to *C. sinensis*, *C. limon*, and *C. reticulata*, with mean lesion lengths on these hosts ranging from 5 to 7 cm. Only *Do. viticola* and *N. parvum* were found among the *Botryosphaeriaceae* in Italy. Specifically, *Do. viticola* was isolated from twig dieback of *Citrus sinensis*, while *N. parvum* was isolated from stem necroses in *C. sinensis* × *Poncirus trifoliata*, commonly used as rootstock, and from trunk cankers in *Microcitrus australasica*, a citrus-related species belonging to *Rutaceae*.

Figure 8. Disease symptoms caused by *Botryosphariaceae* on grapevine, olive and citrus in field-grown plants: (a) dieback in the host canopy; (b and c) canker and discoloured internal tissues occurred on grapevine stem; (d and e) canker and subcortical discoloured tissues on olive branches; (f) olive rotted drupes, and (g) emerging perithecia by bark from an affected olive trunk; (h) trunk canker, bark cracking and gummosis on lemon.

PATHOGEN PRODUCTION OF METABOLITES

Interest in phytotoxic metabolites (PMs) produced by *Botryosphaeriaceae* species has increased due to sever impacts of the diseases caused by these fungi in agriculture and forestry (Masi *et al*., 2021; Salvatore *et al*., 2021). Phytotoxins are involved in several diseases, contributing to decline, dieback and specific foliar symptoms (Masi *et al*., 2018a). Phytotoxic metabolites are usually characterized in two main groups: host-selective toxins (HSTs) and non-host-selective toxins (NHSTs) (Pusztahelyi *et al*., 2015). Most PMs produced by *Botryosphaeriaceae* are NHSTs, but an HST named Fraxitoxin (isochromanone) active on ash has been isolated from the emerging pathogen *D. fraxini* (Cimmino *et al*., 2017).

Beyond phytotoxic activity, several secondary metabolites produced by *Botryosphaeriaceae* possess other biological activities, including antifungal, anticancer, antibacterial or insecticidal activities, which make these metabolites promising for different biotechnological applications (Masi *et al*., 2018a). Ability to produce structurally diverse bioactive secondary metabolites in liquid culture has been recognized for several *Botryosphaeriaceae* spp. in *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum* and *Sardiniella* (Andolfi *et al*., 2012, 2014a, 2014b; Cimmino *et al*., 2019; Reveglia *et al*., 2020). Among the most interesting metabolites produced by *Botryosphaeriaceae*, the tetracyclic pimarane diterpene Sphaeropsidin A (SphA) has broad spectrum of activity, for potential applications in agriculture and medicine. Cytotoxicity of SphA, towards apoptosis- and multidrug-resistant cancers, is of particular interest (Mathieu *et al*., 2015; Masi and Evidente, 2021). SphA and several analogues is produced *in vitro* by different pathogenic *Diplodia* spp. and particularly by the oak pathogen *D. quercivora* (Andolfi *et al*., 2014b). Recent advances regarding bioactive secondary metabolites produced by *Botryosphaeriaceae* spp. are reported in Table 2.

BOTRYOSPHAERIACEAE TAXONOMY AND IDENTIFICATION

Systematics and taxonomy have been revisited and updated according to the newest molecular evidence, which has clarified the phylogenetic relationships of several cryptic species (Crous *et al*., 2006; Alves *et al*., 2008; Phillips *et al*., 2013). In addition to morphological data, phylogenetic analyses based on concatenated ITS and *tef1* sequence data are usually used for the identification and description of new putative species (Alves *et al*., 2014; Linaldeddu *et al*., 2015a), and sequences of the LSU, ITS, *tef1* and *tub2* regions have been used for delimitation or description of new genera (Phillips *et al*., 2008, 2019; Linaldeddu *et al*., 2016a). The rapid increase in the number of newly described *Botryosphaeriaceae* spp. shows that sequence data analyses for species identification in some genera such as *Diplodia* and *Neofusicoccum* is becoming increasingly difficult (Lopes *et al*., 2017, 2018).

Incorrect analysis of nucleotide sequences or in choice of the gene region to use in phylogenetic analysis exposes risk that new names are assigned to species haplotypes, rather than to new biologically distinct species. For example, Linaldeddu *et al*. (2013), revealed the existence of two distinct haplotypes in *D. corticola*, named A and B, based on nine fixed differences in the sequences of the *tef1* region. The intraspecific variability in some housekeeping genes of *Botryosphaeriaceae* raises doubts about the limits of multilocus sequence analyses for accurate species delimitation (Lopes *et al*., 2017, 2018). For *D. corticola*, the *tef1* locus should be used to study phylogeographic diversity among countries, but not for discrimination of closely related species (Smahi *et al*., 2017; Lopes *et al*., 2018). For *Neofusicoccum* and *Diplodia*, *MAT* genes have been shown to be the excellent phylogenetic markers, giving capacity to identify and delimit cryptic species (Lopes *et al*., 2017, 2018). Using *MAT* genes together with ITS regions for the description of new *Diplodia* and *Neofusicoccum* species has considerable potential.

CHALLENGES AND FUTURE PERSPECTIVES

In depth observations of economically and ecologically important agricultural crops and forest plantation trees in Italy have led to the discovery of the high diffusion of some destructive diseases caused by *Botryosphaeriaceae*. These plant pathogens are becoming limiting factors for plant production, reducing yields, product quality, and profitability (Carlucci *et al*., 2015; Linaldeddu *et al*., 2016a, 2016b, 2016c; Gusella *et al*., 2020b; Aiello *et al*., 2022; Guarnaccia *et al*., 2022). Pathogen spread and infection development could occur during any of the crop cultivation steps. However, host plant propagation processes and grafting are key steps for obtaining and producing healthy plants. Disease symptoms observed during the first years after transplanting often reveal the presence of previous fungal infections which have occurred in the nurseries, especially during these propagation steps (Aiello *et al*., 2020; Costanzo *et al*., 2022). Use of certified propagation material and an early detection are then required to limit potential damage caused by these fungal diseases. A key challenge in

Table 2. Secondary metabolites produced by *Botryosphaeriaceae*, and their biological activities.

Secondary metabolite	Species	Biological activity	References
(1R,2R)-jasmonic acid	*Lasiodiplodia mediterranea*	Phytotoxic	Andolfi *et al.*, 2014a
(+)-epi-Epoformin	*Diplodia quercivora*	Antifungal, antioomycetes, phytotoxic	Andolfi *et al.*, 2014b
Afritoxinone A	*Diplodia africana*	Phytotoxic	Evidente *et al.*, 2012
Afritoxinone B	*Diplodia africana*	Phytotoxic	Evidente *et al.*, 2012
Botryosphaerone D	*Neofusicoccum australe*	Phytotoxic	Andolfi *et al.*, 2012
Cyclobotryoxide	*Neofusicoccum cryptoaustrale*	Phytotoxic	Andolfi *et al.*, 2012
Diplopyrone	*Diplodia corticola*	Phytotoxic	Evidente *et al.*, 2003a Masi *et al.*, 2016
Diplopimarane	*Diplodia quercivora, Diplodia olivarum*	Antifungal, antioomycetes, phytotoxic, zootoxic	Andolfi *et al.*, 2014b; Di Lecce *et al.*, 2021
Diorcinol	*Diplodia corticola*	Phytotoxic, antifungal, antioomycetes, zootoxic	Cimmino *et al.*, 2016 Masi *et al.*, 2016
Diplopyrone B	*Diplodia corticola*	Antifungal, antioomycetes, phytotoxic	Cimmino *et al.*, 2016 Masi *et al.*, 2016
Diploquinones A	*Diplodia mutila*	Phytotoxic	Reveglia *et al.*, 2018a, 2019
Diploquinones B	*Diplodia mutila*	Phytotoxic	Reveglia *et al.*, 2018a, 2019
Epi-Sphaeropsidone	*Diplodia africana, Diplodia cupressi, Diplodia subglobosa*	Antifungal, phytotoxic	Evidente *et al.*, 2012; Masi *et al.*, 2022
Fraxitoxin	*Diplodia fraxini*	Phytotoxic	Cimmino *et al.*, 2017
Luteoethanones A	*Neofusicoccum luteum*	Phytotoxic	Masi *et al.*, 2021
Luteoethanones B	*Neofusicoccum luteum*	Phytotoxic	Masi *et al.*, 2021
Neoanthraquinone	*Neofusicoccum luteum*	Phytotoxic	Masi *et al.*, 2020a
Olicleistanone	*Diplodia olivarum*	Zootoxic	Di Lecce *et al.*, 2021
Oxysporone	*Diplodia africana*	Phytotoxic	Evidente *et al.*, 2012
Pinofuranoxins A	*Diplodia sapinea*	Antifungal, phytotoxic zootoxic	Masi *et al.*, 2021
Pinofuranoxins B	*Diplodia sapinea*	Antifungal, antioomycetes, phytotoxic, zootoxic	Masi *et al.*, 2021
R-(-)-Mellein	*Diplodia africana, Sardiniella urbana*	Antifungal, phytotoxic	Cimmino *et al.*, 2019
Resorcinol	*Dothiorella vidmadera*	Phytotoxic	Reveglia *et al.*, 2018b
Sapinofuranone A	*Diplodia sapinea*	Phytotoxic	Evidente *et al.*, 1999
Sapinofuranone B	*Diplodia sapinea*	Phytotoxic	Evidente *et al.*, 1999
Sapinopyridione	*Diplodia sapinea*	Phytotoxic, Antifungal	Evidente *et al.*, 2006
Spencertoxin	*Dothiorella viticola*	Phytotoxic	Reveglia *et al.*, 2020
Sphaeropsidin A	*Diplodia africana, Diplodia cupressi, Diplodia corticola, Diplodia olivarum, Diplodia quercivora, Diplodia subglobosa*	Phytotoxic, antifungal, antioomycetes, antibacterial, anticancer, insecticidal, zootoxic	Andolfi *et al.*, 2014b; Masi *et al.*, 2016, 2022; Masi and Evidente, 2021; Di Lecce, 2021; Salvatore *et al.*, 2021; Roscetto *et al.*, 2020
Sphaeropsidin B	*Diplodia cupressi*	Phytotoxic, Antifungal	Evidente *et al.*, 1997, 2011
Sphaeropsidin C	*Diplodia corticola, Diplodia cupressi, Diplodia olivarum, Diplodia quercivora*	Phytotoxic	Evidente *et al.*, 1997; Andolfi *et al.*, 2014b; Di Lecce, 2021; Masi *et al.*, 2016
Sphaeropsidin D	*Diplodia cupressi*	Phytotoxic	Evidente *et al.*, 2002
Sphaeropsidin F	*Diplodia cupressi*	Phytotoxic	Evidente *et al.*, 2003b
Sphaeropsidin G	*Diplodia corticola, Diplodia olivarum*	Zootoxic	Cimmino *et al.*, 2016; Di Lecce, 2021
Sphaeropsidone	*Diplodia cupressi*	Phytotoxic	Evidente *et al.*, 1998

the knowledge of *Botryosphaeriaceae* involves developing tools that provide rapid identification of fungi in asymptomatic plants, particularly in planting material. High throughput sequencing (HTS) diagnostics are important advances in plant pathology, as key molecular biology contributions since the development of the PCR process

(Robert-Siegwald *et al*., 2017). In addition, image analysis is a promising technique among non-invasive detection techniques for *Botryosphaeriaceae* spp. Hyper- or multi-spectral image analysis allows diagnosis of wood diseases of symptomatic and asymptomatic grapevine plants, even before disease symptoms appear (Pérez-Roncal *et al*., 2022). Unmanned aerial vehicles could be used to monitor entire orchards (Di Gennaro *et al*., 2016), and future studies should focus on these, aiming to enable early disease detection and improve plant protection management processes.

To decrease the use of synthetic fungicides on crops, cultural practices to manage *Botryosphaeriaceae* diseases (elimination of dead wood or pruning residues to reduce potential inoculum sources) must be complemented by employing biological control measures for pruning wound protection, such as microbial active ingredients or substances of botanical origin (essential oils, wood extracts) (Špetík *et al*., 2022), or chemio-physical tools (Baaijens *et al*., 2019). As outlined above, global warming and stress factors such as occasional climatic events or inappropriate agronomic management may further compromise plant health, and can affect susceptibility host plants and behaviour of pathogen, increasing infection risk (Batista *et al*., 2021). The use of windbreaks, precision irrigation, anti-frost irrigation, and soil mulching, may also be useful for the pathogen management by reducing host stress.

Further research should aim to ascertain the epidemiology of these pathogens in Italian nurseries, forestry and orchards, and to evaluate the risks of fungal infections over time in different global climate change scenarios. Decision-support systems develop models to predict species occurrence in time and space (Batista *et al*., 2023), and these can support grower choices for determining the best times for disease management intervention. This research will help to reduce the impacts of ubiquitous *Bortryosphaeriacea* as pathogens of economically and aesthetically important plants.

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Research Papers

Cercospora leaf spot of olive in Uruguay

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Summary. Cercospora Leaf Spot (CLS) of olive is an important fungal disease in Uruguay, causing severe early defoliation. Fungal isolates were obtained from olive leaves with typical CLS symptoms from Uruguayan orchards. The isolates were identified based on phenotypic characteristics and DNA sequence analyses. Infection processes under field conditions were characterized. Phylogenetic analyses confirmed that *Pseudocercospora cladosporioides* is the causal agent of CLS in Uruguay. Three colony morphologies were observed for isolates growing on potato dextrose agar. Mean conidium length ranged from 65.7 to 101.8 μm, and widths from 4.3 to 5.0 μm. Mean optimum growth temperature was 21.5°C (range 19.2 to 24.8°C). Under field conditions, initial CLS symptoms on leaves were observed 5 months after inoculation of cv. Arbequina plants, confirming the disease's lengthy incubation period. This study shows that CLS as one of the most prevalent and destructive olive diseases in Uruguay, and emphasizes the importance of further research to develop efficient management of this disease.

Keywords. 'Arbequina', etiology, *Olea europaea, Pseudocercospora cladosporioides*.

INTRODUCTION

Olive (*Olea europaea* subsp. *europaea* L.) is an important fruit crop in Uruguay, covering around 5,900 ha. The most commonly planted olive cultivars are Arbequina (47%), Coratina (21%), Picual (11%), and Frantoio (10%) (MGAP-DIEA, 2020). The Uruguayan climate is characterized by frequent high humidity days and abundant rainfall, favouring development of fungal leaf and fruit diseases of olive (Conde-Innamorato *et al*., 2019).

Cercospora leaf spot (CLS) is an endemic and severe olive disease (Del Moral and Medina, 1985) that is widely distributed in most olive-growing areas, causing severe losses during wet years in susceptible cultivars (Trapero *et al*., 2017). However, in the Mediterranean basin region where most olive production is concentrated, little research has focused on this disease. In that region, CLS is considered as less severe than other olive diseases, such as anthracnose or olive scab (Garrido *et al.,* 2022).

CLS causes severe “early leaf drop” defoliation of olive trees. Affected leaves have diffuse and chlorotic areas on the adaxial surfaces which evolve necrotically, and leaden-grey areas on the abaxial surfaces due to the presence of conidia. Host petioles, peduncles (Ávila *et al.,* 2004; Agustí-Brisach *et al.,* 2016), and young twigs can also be affected, where blackened spots of different shapes and sizes can be observed (Pappas, 1993; Nigro and Ferrara, 2011). Olive fruit can also be affected, and symptoms vary from brown, sunken areas of a few millimetres diam. on green olives to more extensive areas with pale yellow haloes on ripening fruit. Severe symptoms cause decreases in fruit quality and oil production, due to fruit drop, increased acidity, and reduced oil yields (Trapero *et al.*, 2017; Avila *et al.*, 2020, Romero *et al.*, 2020).

The causal agent of CLS is the fungus *Pseudocercospora cladosporioides*, which is characterized by slow growth in culture media and little or no production of conidia (Pappas, 1993; Ávila *et al.*, 2004, 2005, 2020; Nigro and Ferrara, 2011). Conidia of the fungus are produced in dark brown stromatic conidiomata, which arise in clusters through the host stomata or directly through the epidermis on the underside of infected leaves (Ávila *et al.*, 2004). Conidia are pale brown, straight or slightly curved, rounded at the apices and truncated at the bases, with variable dimensions and numbers of septa (Sarasola, 1951; Del Moral and Medina, 1985; McKenzie, 1990; Braun, 1993; Ávila *et al.*, 2004; Sergeeva *et al.*, 2008; Nigro and Ferrara, 2011). Little is known of the CLS disease cycle, except that the main inoculum source is affected leaves that remain attached to tree, and that the disease incubation period is long (up to 11 months) (Ávila *et al.*, 2004; Sergeeva and Spooner-Hart, 2009; Agustí-Brisach *et al.*, 2016; Trapero *et al.*, 2017; Ávila *et al.*, 2020).

In Uruguay, Conde-Innamorato *et al.* (2013) found that CLS was one of the main foliar diseases affecting olive trees. However, farmers often lack awareness of this disease mistaking CLS for other foliar diseases such as olive scab or anthracnose, as well as symptoms caused by abiotic factors. Developing local knowledge of CLS is urgent to elucidate aspects of the disease that facilitate understanding the interactions between host plants, the pathogen, and the environment, and to develop effective control strategies. For this reason, the research outlined in this paper aimed to characterize the causal agent of CLS of olive in Uruguay using morphological and molecular analyses, and to characterize the infections process under field conditions.

MATERIALS AND METHODS

Field symptoms and fungal isolates

Between 2017 and 2018, a survey was conducted in 18 olive orchards situated in six departments located in the north (Salto), south (Colonia, Canelones, and Montevideo) and east (Maldonado and Rocha) of Uruguay (Table 1). The cultivars sampled were Arbequina, Arbosana, Coratina, Leccino, Manzanilla de Sevilla, Pendolino, Picholine, and Seggianese. In each orchard, symptoms attributable to CLS were carefully observed, and five to ten leaves with typical CLS symptoms were collected from different trees and used for pathogen isolations. From each leaf, the sporulating lesion was hydrated with 300 μL of sterile distilled water (SDW), and 100 μL of the conidium suspension were dispersed in each of 90 mm diam. Petri plates containing water agar (WA) amended with 0.4 g L^{-1} of streptomycin sulphate (Sigma-Aldrich). After incubation for 24 h at 20°C in darkness, germinated conidia were transferred to a Potato Dextrose Agar (PDA, Oxoid Ltd.) and maintained under the same incubation conditions. A single monoconidial isolate was selected from each leaf sample.

The isolates were conserved in 15% glycerol at -80°C, and deposited at the fungal culture collection of the Department of Plant Protection, Faculty of Agronomy, University of the Republic, Uruguay.

Morphological characterization of isolates

Monosporic isolates were grown on PDA at 20°C, in darkness. After 30 d, the isolates were grouped in morphotypes according to colony appearance, shape, and colour. Monosporic isolates growing on Cornmeal Agar (CMA) in the same conditions were used for conidium characterization. Lengths, widths, and the numbers of septa from 20 conidia per isolate were assessed using a Dino Capture 2.0 digital imaging camera (Dino-Eye AM4023X) on an Eclipse E100Led microscope (Nikon Corp.) at ×400 magnification. Data of conidium lengths, widths, length/width ratios, and numbers of septa were subjected to analysis of variance (ANOVA) and Tukey’s test (at $P = 0.05$) was used to compare the mean conidium values. These analyses were conducted using the RStudio v. 2023.06.1-524 program (https://dailies.rstudio.com/version/2023.06.1+524/).

Effects of temperature on isolate mycelium growth

Agar plugs (5 mm diam.) from the outer edges of 15-d-old cultures of isolates were transferred to the cen-

Table 1. Location details and Genbank accession numbers for Uruguayan *Pseudocercospora cladosporioides* isolates obtained from olive leaves, and identified in this study.

Isolate	Orchard	Cultivar[a]	Department, Locality	Morphotype[b]	GenBank Accession No.		
					ACT	CAL	ITS
E07	1	Arbequina	Salto, Olivares Salteños	a	ON442427	ON442509	ON442468
E10	1	Arbequina	Salto, Olivares Salteños	a	ON442428	ON442510	ON442469
E12	2	Arbequina	Salto, Olivares Salteños	a	ON442429	ON442511	ON442470
E15	2	Arbequina	Salto, Olivares Salteños	a	ON442430	ON442512	ON442471
E19	3	n/d	Salto, Punta de Valentín	c	ON442431	ON442513	ON442472
E20	3	n/d	Salto, Punta de Valentín	a	ON442432	ON442514	ON442473
E23	3	n/d	Salto, Punta de Valentín,	a	ON442433	ON442515	ON442474
E25	4	n/d	Salto, Punta de Valentín,	a	ON442434	ON442516	ON442475
E27	4	n/d	Salto, Punta de Valentín,	a	ON442435	ON442517	ON442476
E29	4	n/d	Salto, Punta de Valentín,	a	ON442436	ON442518	ON442477
E31	5	Arbequina	Rocha, Nuevo Manantiales	a	ON442437	ON442519	ON442478
E33	5	Arbequina	Rocha, Nuevo Manantiales	a	ON442438	ON442520	ON442479
E35	6	Coratina	Rocha, Nuevo Manantiales	a	ON442439	ON442521	ON442480
E37	6	Coratina	Rocha, Nuevo Manantiales	a	ON442440	ON442522	ON442481
E39	6	Coratina	Rocha, Nuevo Manantiales	b	ON442441	ON442523	ON442482
E40	6	Coratina	Rocha, Nuevo Manantiales	c	ON442442	ON442524	ON442483
E43	7	Manzanilla	Maldonado, Agroland	a	ON442443	ON442525	ON442484
E48	8	Leccino	Maldonado, Agroland	c	ON442444	ON442526	ON442485
E49	9	Coratina	Maldonado, Agroland	a	ON442445	ON442527	ON442486
E50	9	Coratina	Maldonado, Agroland	a	ON442446	ON442528	ON442487
E51	9	Coratina	Maldonado, Agroland	a	ON442447	ON442529	ON442488
E52	9	Coratina	Maldonado, Agroland	a	ON442448	ON442530	ON442489
E53	10	Arbequina	Maldonado, Agroland	a	ON442449	ON442531	ON442490
E58	11	Arbequina	Montevideo, ARU	a	ON442450	ON442532	ON442491
E59	11	Arbequina	Montevideo, ARU	c	ON442451	ON442533	ON442492
E60	11	Arbequina	Montevideo, ARU	a	ON442452	ON442534	ON442493
E66	12	Pendolino	Montevideo, ARU	a	ON442453	ON442535	ON442494
E68	12	Pendolino	Montevideo, ARU	a	ON442454	ON442536	ON442495
E69	12	Pendolino	Montevideo, ARU	a	ON442455	ON442537	ON442496
E70	13	Leccino	Canelones, INIA Las Brujas	b	ON442456	ON442538	ON442497
E71	13	Leccino	Canelones, INIA Las Brujas	b	ON442457	ON442539	ON442498
E72	14	Picholine	Canelones, INIA Las Brujas	a	ON442458	ON442540	ON442499
E73	15	Seggianese	Canelones, INIA Las Brujas	a	ON442459	ON442541	ON442500
E74	15	Seggianese	Canelones, INIA Las Brujas	b	ON442460	ON442542	ON442501
E76	16	n/d	Montevideo, FAgro	a	ON442461	ON442543	ON442502
E77	16	n/d	Montevideo, FAgro	a	ON442462	ON442544	ON442503
E78	17	Arbequina	Colonia, San Pedro	a	ON442463	ON442545	ON442504
E79	17	Arbequina	Colonia, San Pedro	c	ON442464	ON442546	ON442505
E82	18	Arbosana	Colonia, Astilleros,	a	ON442465	ON442547	ON442506
E83	18	Arbosana	Colonia, Astilleros	a	ON442466	ON442548	ON442507
E85	15	Seggianese	Canelones, INIA Las Brujas	a	ON442467	ON442549	ON442508

[a] n/d: not determined
[b] Morphotype: a, grey and rough; b, whitish and rough; c, grey olivaceous and smooth.

tres of the fresh PDA plates. The plates were then incubated in darkness at different temperatures from 0°C to 35°C at 5°C increments. For each combination of isolate and temperature, three replicates plates were used, and the experiment was performed twice. After 30 d, each colony diameter was measured along two perpendicular

Table 2. Details of primers used in this study for amplification and sequencing.

Locus	Primer	Sequence (5’-3’)	Orientation	Annealing	Reference
ITS	ITS1	TCCGTAGGTGAACCTGCGG	Forward	57°C for 30 s	White *et al.* (1990)
	ITS4	TCCTCCGCTTATTGATATGC	Reverse		
ACT	ACT-512F	ATGTGCAAGGCCGGTTTCGC	Forward	52°C for 30 s	Carbone and Kohn (1999)
	ACT-783R	TACGAGTCCTTCTGGCCCAT	Reverse		
CAL	CAL-228F	GAGTTCAAGGAGGCCTTCTCCC	Forward	52°C for 30 s	Carbone and Kohn (1999)
	CAL-737R	CATCTTTCTGGCCATCATGG	Reverse		

axes, using a digital calliper (IP54; Truper Tools). These colony dimensions were averaged, and the radial growing rate (mm day^{-1}) was calculated.

To examine fluctuations in mycelial growth rates across different temperatures for each isolate, a non-linear data adjustment method was employed, using the Generalized Analytis Beta model (Hau and Kranz, 1990; López-Moral *et al*., 2017). Subsequently, the optimum growth temperature (*Topt*) was determined using the formula $Topt = [(a \times Tmax) + (b \times Tmin)] / (a + b)$ and the corresponding maximum growth rate (MGR) was calculated using the equation $Y = d \times (T - Tmin)^a \times (Tmax - T)^b$. Data analyses were conducted using Statistix 10 (Analytical Software, 2013). Ten representative isolates were selected according to geographic origin, optimum growth temperature, and daily radial growth rate at the optimum temperature, according to non-linear model results, and subjected to ANOVA analysis. Tukey’s test (at $P = 0.05$) was used to compare the mean growth rates. These analyses were carried out using the RStudio v. 2023.06.1-524 program.

Molecular characterization of isolates

DNA extraction, PCR analysis and sequencing

DNA was extracted from the mycelium of each monosporic isolate following the protocol of Paolocci *et al*. (1999). Three genomic regions of each isolate were amplified, including the ITS region (ITS), using ITS1/ITS4 primers (White *et al*., 1990), portions of actin (ACT), using ACT-512F/ACT-783R primers (Carbone and Kohn 1999), and calmodulin (CAL), using CAL-228F/CAL-737R primers (Carbone and Kohn 1999) (Table 2).

Each PCR reaction contained 1× PCR buffer, 2.5 mM MgCl2, 0.4 mM of each dNTP, 0.4 μM of each primer, 0.5 U of DNA polymerase (Bioron), and 1 μL of template DNA. The PCR reactions were each adjusted to a final volume of 20 μL with MQ water. The amplifications were carried out on a MultiGene™ Mini thermal cycler (Labnet International Inc.). The PCR program consisted on an initial step of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for ITS, and 52°C for ACT and CAL for 30 s, and elongation at 72°C for 45 s. The final extension was at 72°C for 10 min. PCR products were analyzed on 1.5% agarose gels stained with GelRed™, and were visualized in a transilluminator under UV light. A GeneRuler 100-bp DNA ladder plus (Thermo) was used as the molecular weight marker. PCR products were purified and sequenced at the Pasteur Institute, Montevideo, Uruguay.

Phylogenetic analyses

The sequences of each gene region were aligned using ClustalW, available within the MEGA v. 11.0.11 program (Tamura *et al*., 2021). The sequences were compared with those deposited in NCBI GenBank nucleotide database (www.ncbi.nim.nih.gov) using the BLAST source. Sequences of phylogenetically related species of *P. cladosporioides* (including the ex-epitype, CBS 117482) of the *Pseudocercospora* phylogenetic analysis Clade 14 (Crous *et al*., 2013) were obtained from GenBank and incorporated into the alignments (Table 3).

Phylogenetic analyses were carried out separately for each ITS, ACT, and CAL region, and a multi-locus alignment was built using Concatenate Sequence Alignments available within the MEGA v. 11.0.11 program. Phylogenetic trees were constructed using Bayesian Inference (BI) with the MrBayes v. 3.2.7 program, and Maximum Likelihood (ML) with the RAxML v. 8.2.12 program, implemented in CIPRES Science Gateway v. 3.3 (http://www.phylo.org/). For BI phylogenetic analyses, the best-fit model of each gene region in each genus was selected, according to the corrected Akaike information criteria (cAIC) in MEGA v. 11.0.11, and Jukes Cantor (JC) resulted as the best model for the three gene regions. Four Marko Chain Monte Carlo (MCMC) chains were run simultaneously starting from a random

Table 3. GenBank sequences used in this study for phylogenetic analyses of representative fungal isolates.

Species [a]	Strain [a,b]	Host	Origin country	Collector	GenBank Accession No.[c]		
					ITS	ACT	CAL
Cercospora sojina	**CBS 132615 = CPC 11353**	*Glycine soja*	South Korea	H.D. Shin	JX143659	JX143173	JX142927
Pseudocercospora araliae	CPC 10154	*Aralia elata*	South Korea	H.D. Shin	GU269652	GU320360	-
P. araliae	**MUCC 873**	*Aralia elata*	Japan	T. Kobayashi and C. Nakashima	GU269653	GU320361	-
P. balsaminae	CBS 131882 = CPC 10044	*Impatiens textorian*	South Korea	H.D. Shin	GU269660	GU320367	-
P. boehmeriigena	CPC 2524 = COAD 1562	*Bohemia nivea*	Brazil	R.W.Barreto	KT290152	KT313507	-
P. cladosporioides	CBS 113866	*Olea europaea*	Spain	A. Ávila et al.	AY438252	AY438244	AY438261
P. cladosporioides	CBS 113867	*Olea europaea*	Spain	A. Ávila et al.	AY438254	AY438246	AY438263
P. cladosporioides	CBS 114079	*Olea europaea*	Spain	A. Ávila et al.	AY438249	AY438241	AY438258
P. cladosporioides	**CBS 117482 = CPC 10913**	*Olea europaea*	Tunisia	P.W. Crous	GU269678	GU320383	DQ008124
P. crocea	**CBS 126004 = CPC 11668**	*Pilea hamaoi*	South Korea	H.D. Shin	GU269792	GU320493	-
P. dendrobii	MUCC 596	*Dendrobium* sp.	Japan	C. Nakashima and K. Motohashi	GU269696	GU320401	-
P. dianellae	CBS 117746	*Dianella caerulae*	New Zealand	C.F. Hill	GU269695	GU320400	-
P. eucalyptorum	**CBS 110777 = CPC 16 = CMW 5228**	*Eucalyptus nitens*	South Africa	P.W. Crous	AF309598	KF903406	KF902621
P. eucalyptorum	CPC 12406 = CBS 132029	*Eucalyptus globulus*	Australia	I. Smith	GU269793	GU320494	KF902616
P. gracilis	CBS 111189 = CPC 1315	*Eucalyptus urophylla*	Indonesia	M.J. Wingfield	DQ302960	JX902137	JX901572
P. gracilis	**CBS 242.94 = CPC 729**	*Eucalyptus urophylla*	Indonesia	P.W. Crous	DQ267582	DQ147616	-
P. humulicola	CBS 131585 = CPC 11358	*Humulus scandens*	South Korea	H.D. Shin	GU269723	GU320427	-
P. humulicola	CBS 131883 = CPC 10049	*Humulus scandens*	South Korea	H.D. Shin	GU269724	JQ325018	-
P. jussiaeae	CBS 132117 = CPC 14625	*Ludwigia prostrata*	South Korea	H.D. Shin	JQ324977	JQ325020	-
P. lythri	CBS 132115 = CPC 14588	*Lythrum salicaria*	South Korea	H.D. Shin	GU269742	GU320444	-
P. lythri	**MUCC 865**	*Lythrum salicaria*	Japan	I. Araki and M. Harada	GU269743	GU320445	-
P. nephrolepidis	CBS 119121	*Nephrolepis auriculata*	Taiwan	R. Kirschner	GU269751	GU320453	-
P. plectranthi	**CBS 131586 = CPC 11462**	*Plectranthus* sp.	South Korea	H.D. Shin	GU269791	GU320492	-
P. pouzolziae	CBS 122280	*Gonostegia hirta*	Taiwan	R. Kirschner	GU269761	GU320462	-
P. profusa	**CBS 132306 = CPC 10055**	*Acalypha australis*	South Korea	H.D. Shin	GU269762	GU320463	-
P. profusa	CPC 10042	*Acalypha australis*	South Korea	H.D. Shin	GU269787	GU320488	-
P. rhabdothamni	**CBS 114872**	*Rhabdothamnus solandri*	New Zealand	M. Fletcher	GU269768	GU320471	-
P. robusta	**CBS 111175 = CPC 1269 = CMW 5151**	*Eucalyptus robur*	Malaysia	M.J. Wingfield	AY309597	DQ147617	JX901579
P. rumohrae	CBS 117747	*Marattia salicina*	New Zealand	C.F. Hill	GU269774	GU320477	-
Pseudocercospora sp.	CPC 10058	*Potentilla kleiniana*	South Korea	H.D. Shin	JQ324979	JQ325022	-

[a] Ex-epitype or holotype species and strain are indicated in bold font.
[b] CBS: Culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria, Pretoria, South Africa; COAD: Coleçao Octavio de Almeida Drumond, housed at the Universidade Federal de Viçosa, Viçosa, Brazil; CPC: Culture collection of Pedro Crous, housed at the Westerdijk Institute; MUCC (in TSU): Culture Collection, Laboratory of Plant Pathology, Mie University, Tsu, Mie Prefecture, Japan.
[c] ITS: internal transcribed spacers; ACT: actin; CAL: calmodulin.

tree to 10 million generations. Trees were sampled every 1000 generations, and the first 2500 were discarded as the burn-in phase of each analysis. Posterior probabilities were determined from a majority-rule consensus tree generated with the remaining 7500 trees. For the ML analyses, a Generalized Time-Reversible with Gamma correction (GTR + GAMMA) nucleotide substitution model and 1000 bootstrap iterations were indicated. The other parameters were used as default settings. Sequences generated in this research were deposited in the GenBank (Table 1).

Characterization of infection under field conditions

To determine the period between inoculation and the onset of visible symptoms, field inoculations were carried out. Two experiments were carried out in 2021, one during autumn, the other in spring. The experiments were conducted on 15-year-old cv. Arbequina olive trees in an experimental orchard at the INIA Las Brujas Agricultural Research Station, Canelones, Uruguay (34°40'S, 56°20'W).

Inoculum used was from naturally infected olive leaves, following the methods outlined by Ávila *et al.* (2020). The inoculum was collected from two orchards situated in Rincón del Colorado, Canelones, one of which contained 'Frantoio' olive trees and the other contained cv. Arbequina trees. To obtain each conidium suspension, 150 leaves with sporulating lesions were placed in an Erlenmeyer flask containing 100 mL of sterile distilled water (SDW) plus a drop of Tween 20. The flask was then shaken for 1 h to dislodge the conidia, and the resulting suspension was filtered through sterile gauze. The concentration of conidia was then adjusted to 1.5×10^5 conidia mL^{-1}, using haematocytometer assessments. To check conidium germination, an aliquot from each suspension was plated on water agar, and germination was evaluated after 24 h. Conidium suspensions with germination greater than 75% were used for inoculations.

Three 15-year-old cv. Arbequina trees were randomly chosen from the experimental orchard. Within each tree, four new shoots were selected, positioned, respectively, in the north (N), south (S), east (E), or west (W) quadrants of the tree, with each shoot containing approx. ten leaves. The shoots were sprayed with conidium suspension until runoff. To establish the baseline level of latent infections at the beginning of the experiment, an additional four shoots in each of the same trees and quadrants were inoculated with SDW plus Tween 20 as experimental controls. Each individual shoot was subsequently enclosed within a white non-textile cloth bag until the conclusion of the experiment, to prevent further natural infections caused by *P. cladosporioides*.

Monthly evaluations were carried out during 1 year after inoculations, to determine the presence or absence of symptoms related to CLS on each leaf of the inoculated and control treatments. Presence of characteristic *P. cladosporioides* conidia was also assessed using microscope examinations.

RESULTS

Field symptoms and fungal isolates

Typical symptoms of CLS were observed in all the surveyed commercial olive groves. Leaf spots were observed mainly in adult leaves in the middle to lower parts of each tree. On the upper surfaces of the affected leaves, the spots were greenish-yellow to yellow with diffuse edges (Figure 1 a), and some leaves completely yellow or with necrotic areas. On the undersides of the leaves, grey areas of fungal sporulation were observed (Figure 1 b), consisting of typical conidiophores and conidia of *P. cladosporioides* (Figure 1, f and g). Fungal sporulation was often observed before symptoms, especially in Frantoio and Picual cultivars, and severe defoliation was often present (Figure 1 c).

No symptoms were observed on olive fruit during the two years of survey, so no isolates were derived from fruit. Only during August 2022 in one southern orchard (Canelones department) were typical symptoms of CLS observed on unharvested 'Coratina' olives 5 months after conventional harvest. Affected olives had irregular purple or light brown spots that progressed into depressed greyish-brown areas. A binocular magnifying glass and microscope examinations (Figure 1, d and e) showed typical conidiomata and conidia of *P. cladosporioides*.

A total of 41 monosporic isolates were obtained from leaves exhibiting CLS symptoms, sourced from the 18 olive orchards, as indicated in Table 1.

Morphological characterization of isolates

The isolates exhibited typical morphological characteristics of *P. cladosporioides*. After 15 d incubation at 20°C, the colonies had smooth and well-defined margins, and moderate aerial mycelium. The colonies were smoke-grey to pale olivaceous-grey, with iron-grey reverse sides.

The colonies were of three morphotypes, based on colour and appearance. Thirty-two isolates were of morphotype a (Figure 2 a), with grey rough colonies with

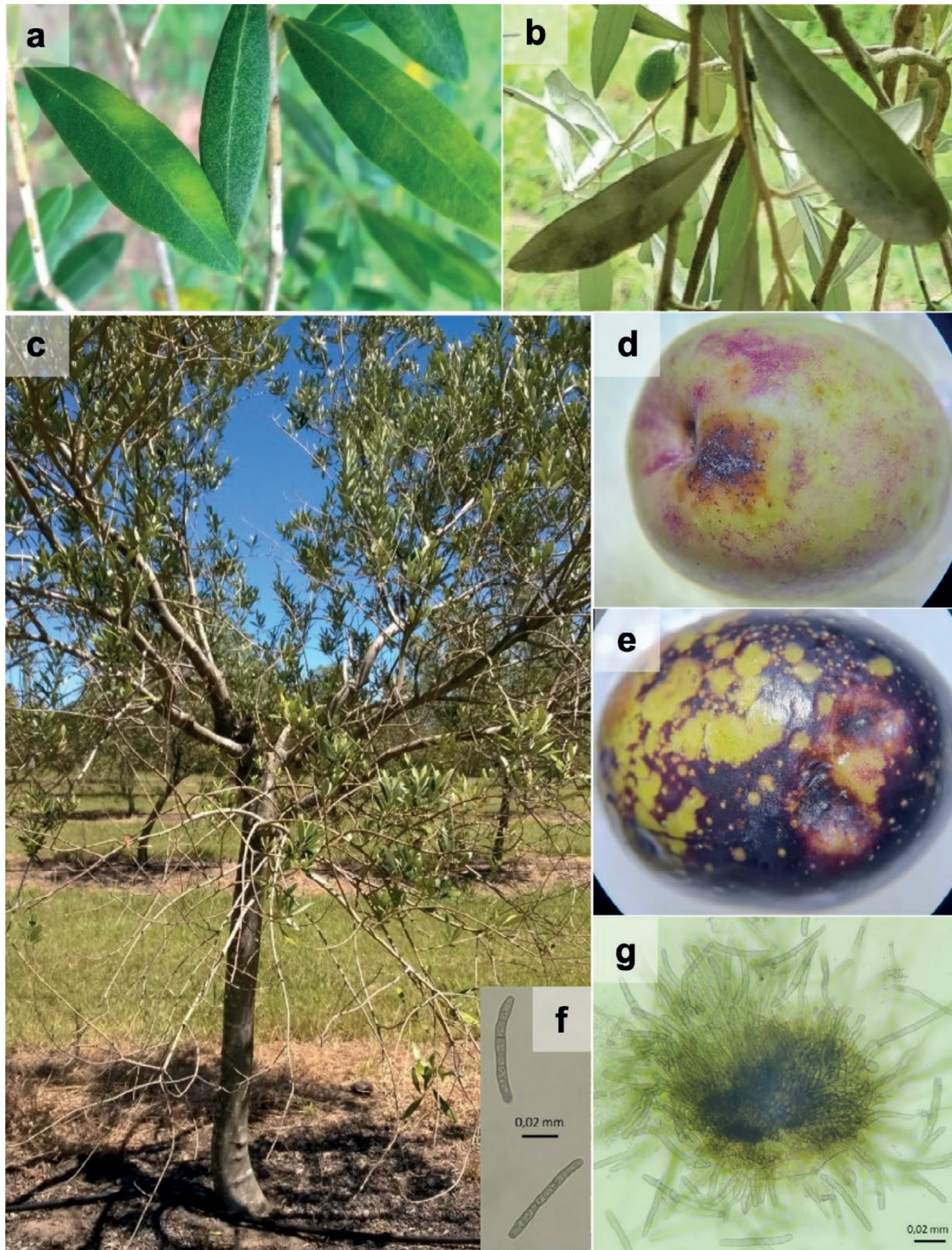

Figure 1. Symptoms of CLS on the olive tree caused by *Pseudocercospora cladosporioides*. a: chlorotic spots on the adaxial surface of the leaves and b: leaden grey areas on the abaxial side due to fructifications of the fungus; c: olive trees with severe defoliation; d and e: fruits with CLS symptoms and reproductive structures of the fungus; f: conidia and g: conidioma of *Ps. cladosporioides*.

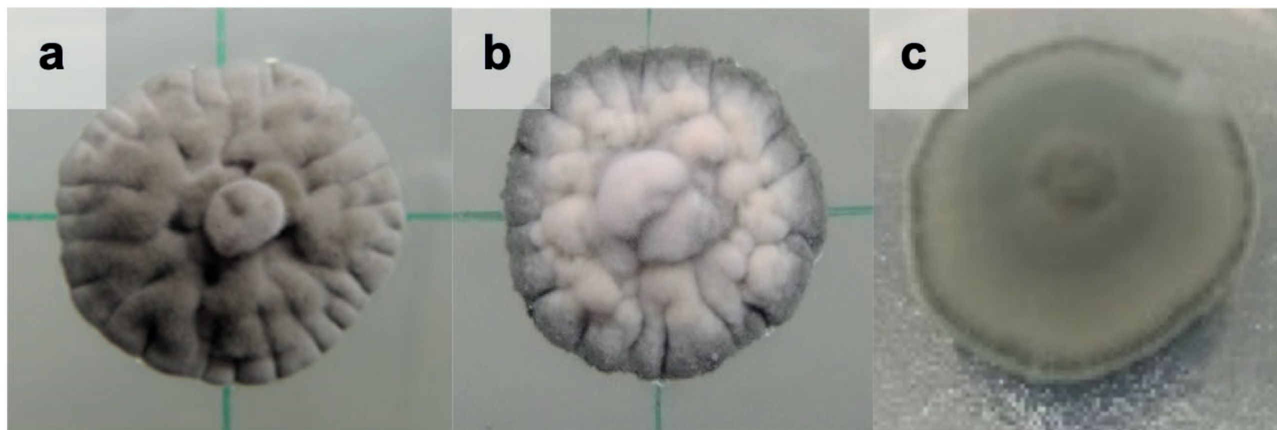

Figure 2. Morphological aspect of the tree morphotype of *Pseudocercospora cladosporioides* colonies growing in PDA culture medium during 15 days at 20ºC in darkness. The three morphotypes are: a: grey and rough; b: whitish and rough and c: grey olivaceous and smooth.

Table 4. Morphological characteristics of conidia of eight representative *Pseudocercospora cladosporioides* isolates on corn meal agar.

Isolate	Colony morphology	Mean conidium dimensions (µm)[a]			Septa
		length (l)	width (w)	ratio l/w	
E19	c	101.8 ± 3.69 a	4.4 ± 0.10 bc	23.1 ± 0.85 a	5.6 ± 0.25 ab
E10	a	94.9 ± 5.83 ab	4.9 ± 0.15 a	19.5 ± 1.35 abc	6.9 ± 0.39 a
E20	a	90.1 ± 3.69 ab	4.4 ± 0.10 bc	20.7 ± 0.85 ab	5.3 ± 0.23 b
E50	a	87.1 ± 3.69 ab	4.9 ± 0.10 a	17.8 ± 0.85 bc	4.8 ± 0.25 b
E74	b	81.2 ± 3.69 bc	4.9 ± 0.10 a	16.7 ± 0.85 cd	4.7 ± 0.24 b
E33	a	80.1 ± 3.69 bc	4.3 ± 0.10 c	18.6 ± 0.85 bc	4.6 ± 0.23 bc
E71	b	75.4 ± 3.69 bc	5.0 ± 0.10 a	15.2 ± 0.85 cd	4.5 ± 0.23 bc
E12	a	65.7 ± 3.69 c	4.9 ± 0.10 a	13.5 ± 0.85 d	3.6 ± 0.24 c
Average		83.7 ± 3.95	4.7 ± 0.11	18.2 ± 0.91	4.8 ± 0.26

[a] The values are means for 20 conidia, ± standard errors. Means in a column followed by the same letter do not differ ($P = 0.05$) according to Tukey's test.

multiple folds. Four isolates were of morphotype b (Figure 2 b), which had light grey to white cottony colonies also with multiple folds. Morphotype c (five isolates; Figure 2 c) had smooth olive to grey colonies (Table 1). In all three groups, the colonies were iron-grey on the reverse sides.

Among all the total isolates examined, only eight had sparse conidium production on CMA, while none produced conidia on PDA. Presence or absence of conidia was not different between the three morphotypes. Conidia were single on each conidiophore, and were light brown. They were subcylindrical with subtruncate basal cells and obtuse apical terminal cells. The conidia ranged from 41 to 133 µm in length (mean = 83.7 µm), and from 4 to 6 µm in width (mean = 4.7 µm), and had average length to width ratio of 18.1 µm. Number of transverse septa in the conidia was from two to eight (Table 4).

Effects of temperature on mycelial growth

Based on the non-linear adjustment estimated according the generalized Analytis Beta model, the optimum mycelial growth temperature for the 41 isolates ranged from 19.2 to 24.8°C, with an average of 21.5°C. The average mycelial growth at 5°C was 0.011 mm day^{-1}, and no isolate grew at 35°C (Figure 3). Statistical analyses conducted for the ten isolates showed differences in optimal growth temperatures and maximum daily radial growth rates. Isolate E35 had the highest optimum growth temperature (24.8°C) which was greater than the other isolates, except for isolate E73 (23.9°C), while isolate E68 had the lowest optimum growth temperature (19.2°C). For daily radial growth rates at the optimum temperatures (Table 5), isolates E19 and E40 had the greatest (respectively, 0.399 and

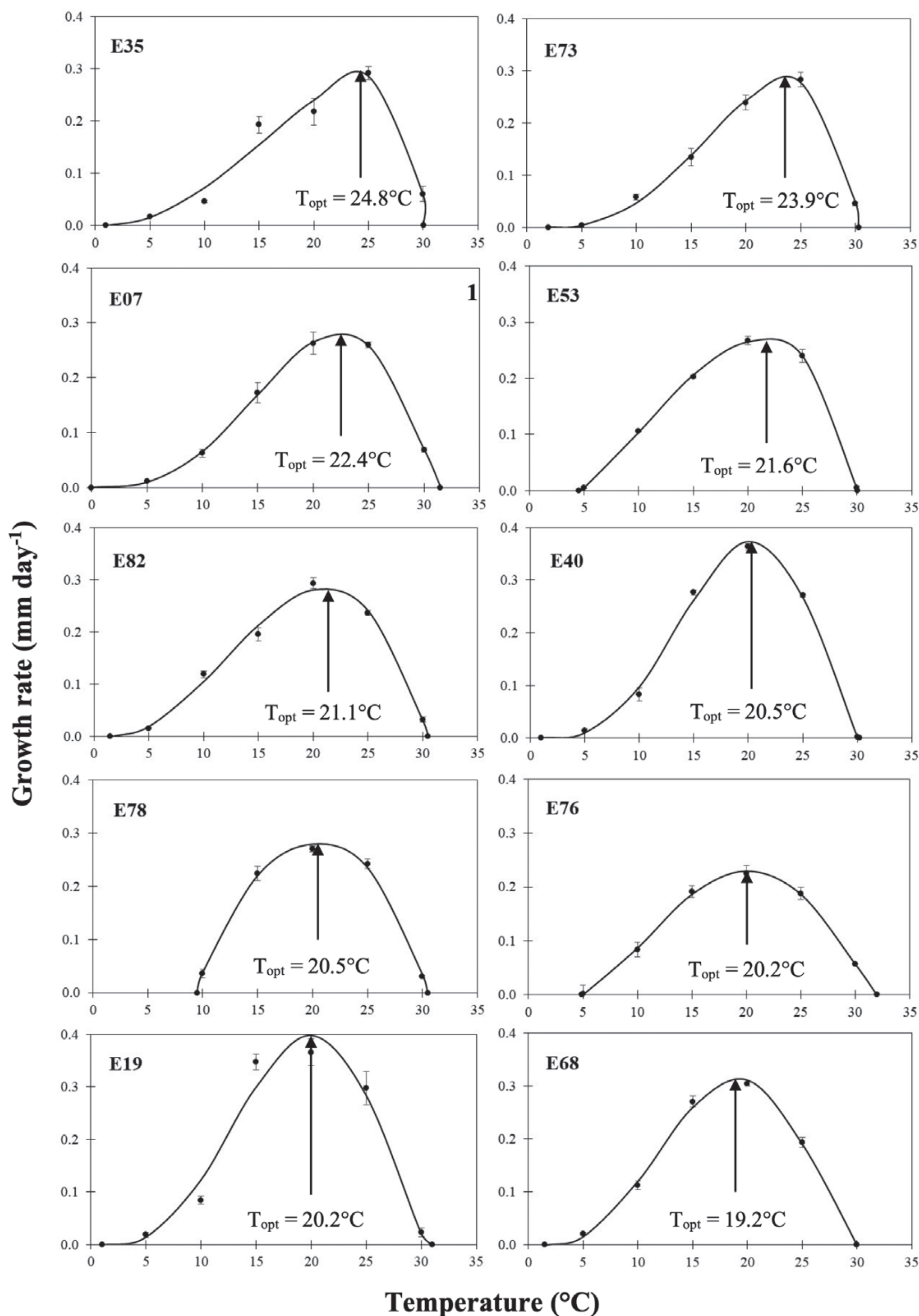

Figure 3. Effect of temperature on mycelial growth rate of a selection of ten *Pseudocercospora cladosporioides* isolates. The isolates were selected according to geographic origin, optimal growth temperature, and daily radial growth rate at the optimum temperature. Isolates were grown on PDA at 0, 5, 10, 15, 20, 25, 30 and 35°C in darkness for 30 days. For each isolate, average growth rates versus temperature were fitted to a nonlinear regression curve using the Analytis Beta model. Data points are the means of two experiments with three replicates per isolate. Vertical bars are standard errors of the mean.

Table 5. Mean temperatures and daily mycelium growth rates for ten representative isolates of ten *Pseudocercospora cladosporioides* isolates. The isolates were grown on PDA at 0, 5, 10, 15, 20, 25, 30 or 35°C in darkness for 30 d.

Isolate	Analytis Beta model[a]			Temperature (°C)[b]			Growth rate[c] (mm.day^{-1})
	R^2	*a*	*b*	Optimum	Minimum	Maximum	
E35	0.9570	1.98	0.42	24.8 a[d]	1.0	30.1	0.288 bc
E73	0.9969	2.69	0.79	23.9 ab	2.0	30.3	0.286 bc
E07	0.9995	3.18	1.29	22.4 bc	0.0	31.5	0.282 bc
E53	0.9998	1.37	0.69	21.6 cd	4.5	30.0	0.270 c
E82	0.9888	2.14	1.03	21.1 cde	1.5	30.5	0.285 bc
E40	0.9954	3.30	1.64	20.5 cde	3.5	30.2	0.374 a
E78	0.9968	0.82	0.74	20.5 de	9.5	30.3	0.281 bc
E76	0.9982	1.58	1.22	20.2 de	4.9	32.0	0.231 d
E19	0.9612	3.08	1.74	20.2 de	1.0	31.0	0.399 a
E68	0.9953	2.67	1.63	19.2 e	1.5	30.0	0.315 b

[a] Analytis Beta model, where R^2 = coefficient of determination, and *a* and *b* = coefficients of regression.
[b] For each isolate, temperature average growth rates were adjusted to the regression curve to optimum growth temperature.
[c] Growth rates at the optimum temperature.
[d] Means in each column followed by the same letter do not differ (P = 0.05), according to Tukey's test.

0.374 mm day^{-1}), while growth rate for isolate E76 was the least (0.231 mm day^{-1}).

Phylogenetic analyses

Preliminary identification based on BLAST search of ITS, ACT, and CAL gene regions, showed high similarity (99 to 100%) of all 41 isolates with the *P. cladosporioides* fungal sequences available in the GenBank Database, including the ex-epitype. The individual sequence datasets showed no significant conflicts in tree topology, indicating that the three genes could be combined. The multiple locus data matrix contained 71 taxa (41 from this study) and 933 characters, including gaps (ITS 1 - 462, ACT 463 - 655, and CAL 656 - 933), of which 75 were parsimony informative.

The tree topologies inferred from BI and ML analyses were consistent with each other. The BI trees are presented, with the support node values of both phylogenetic methods utilized (Figure 4). The 41 Uruguayan isolates grouped in a separate and robust clade (BI/ML: 1/81) with *P. cladosporioides* isolates, including the ex-epitype (CBS 117482), confirming their identity to this species.

Characterization of infection under field conditions

After 5 or 6 months from leaf inoculation, depending on whether this was conducted during autumn or spring, initial symptoms or signs of CLS became apparent in the leaves of inoculated 'Arbequina' plants. In autumn, disease incidence in the inoculated leaves reached 86%. while in spring this reached 91%. The non-inoculated control leaves had 8% infection in both seasons (Figure 5).

Initially, the infected leaves were indistinguishable from healthy leaves. However, on the undersides of the infected leaves, distinct leaden-coloured areas began to emerge. Subsequently, yellowish and chlorotic regions appeared on the upper leaf surfaces, corresponding to the leaden underside zones. Microscopic examinations confirmed the presence of characteristic *P. cladosporioides* conidia within the leaden-grey areas on the abaxial surfaces of the inoculated leaves.

DISCUSSION

Typical CLS symptoms were observed in all of the commercial olive groves surveyed in different areas of Uruguay. This confirms that CLS is a prevalent disease in Uruguayan olive production, as previously described by Conde-Innamorato *et al.* (2013). The generally humid Uruguayan conditions and the susceptibility to CLS of the olive cultivars planted in this country probably account for this finding. Consequently, CLS can be considered as an endemic disease in Uruguay, as has been reported in Spain (Del Moral and Medina, 1985) and Italy (Nigro *et al.*, 2002).

While typical leaf spot, characterised by greenish-yellow to yellow spots with fuzzy edges on upper leaf

Figure 4. Bayesian inference phylogenetic tree built using the concatenated sequences of the ITS, ACT and CAL genomic regions of 41 Uruguayan isolates obtained from olive leaves with typical Cercospora leaf spot disease and sequences obtained from GenBank (ex-type and epi-type strains indicated in bold). *Cercospora sojina* CBS 132615 was used as an outgroup. Bootstrap support values of posterior probability (PP) and Maximum Likelihood (ML) higher than 0.90 and 70%, are shown at the nodes (PP/ML), respectively. Double hash marks indicate branch lengths shortened at least 2-fold to facilitate visualization. The scale bar represents the estimated number of substitutions per site.

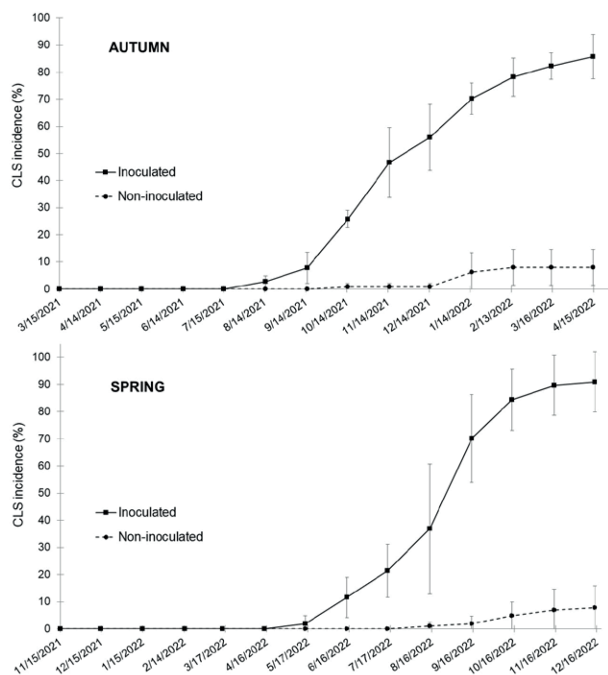

Figure 5. Development of Cercospora Leaf Spot symptoms in 15-year-old olive trees of the Arbequina cultivar inoculated in autumn (03/15/2021) and spring (11/15/2021) (Southern Hemisphere) with a conidial suspension of 1.5×10^5 conidia mL^{-1} of *Pseudocercospora cladosporioides* and evaluated monthly for 13 months.

surfaces, was mainly observed on older leaves (older than 8 months), this symptom was also present on young leaves (4 to 5 months), as was also documented by Nigro *et al.* (2002) and is consistent with the description of Trapero *et al.* (2017). In addition, presence of pathogen structures (*Pseudocercospora* conidia and conidiomata) was verified on the undersides of the leaves, producing leaden grey colouration. As reported by other researchers, the first pathogen signs can anticipate appearance of symptoms on the leaves (Pappas, 1993; Nigro and Ferrara, 2011), as was observed in the present study in Frantoio and Picual cultivars.

The low incidence of CLS symptoms on olive fruit has classified CLS as a foliar disease (Pappas, 1993; Abdelfattah *et al.*, 2015). However, the present study has shown that CLS symptoms on fruit were observed exclusively in one unharvested orchard 5 months after the usual olive harvest date. During autumn, the low to moderate temperatures (10–20°C) accompanied by humid and rainy periods, probably gave favourable conditions for CLS development (Giménez and Castaño, 2013; Ávila *et al.*, 2020). In addition, in Uruguay olive fruits are usually harvested early, between maturity indices of 1 and 2.5, which prioritizes oil quality over yields (Sánchez *et al.*, 2022) and restricts CLS symptom development.

As occurs in Spain (Ávila *et al.*, 2005), the present research showed that *P. cladosporioides* was the sole causal agent of CLS in Uruguay. Multilocus phylogenetic analysis grouped the Uruguayan isolates with the ex-epitype strain of *P. cladosporioides* (CBS 117482), in a well-defined and separate clade to other *Pseudocercospora* species.

Conidium shape, size and number of transverse septae are the most important characters for morphological identification of species of *Pseudocercospora* (Ávila *et al.*, 2004). The Uruguayan isolates showed little or no sporulation on different artificial media, with only a few isolates producing a few conidia on CMA. This low or nil production of conidia in culture has been previously reported (Pappas, 1993; Avila *et al.*, 2004, 2020). In the present study, some conidia were longer than those previously reported for this species (Sarasola, 1951; Del Moral and Medina, 1985; McKenzie, 1990; Pappas, 1993; Avila *et al.*, 2004; Sergeeva *et al.*, 2008). According to Sarasola (1951), these differences can be a consequence of the origins (leaves, fruits, or artificial culture media) of the conidia. The variability of reproductive structures may also be due to the development state of conidia and to environmental conditions (Avila *et al.*, 2020). For example, Pappas (1993) mentioned that formation of large fructifications occurred in humid areas.

Optimum temperatures for mycelium growth varied for the different *P. cladosporioides* from 19.2 to 24.8°C, and maximum colony growth rate was from 0.231 to 0.399 mm day^{-1}. The optimum temperature average for the 41 isolates was 21.5°C. These parameters were similar to those reported by Avila *et al.* (2020) and Pappas (1993), who respectively reported optimum growth temperature for this fungus of 21°C and 22°C. Adaptability of the pathogen to grow in a wide range of temperatures allows it to develop in different environments.

The initial CLS symptoms on field-inoculated cv. Arbequina leaves were visible after 5 to 6 months in spring and autumn, and 11 months after the date of inoculations, infections incidence of approx. 80% was recorded. These results confirm the long incubation period of *P. cladosporioides* under field conditions (Del Moral and Medina, 1985; Trapero *et al.*, 2017; Ávila *et al.*, 2020). CLS symptoms in non-inoculated leaves can also originate from periods preceding inoculations, and these pose challenges for determining if asymptomatic leaves are healthy or are undergoing incubation periods required by this pathogen.

In conclusion, this study has confirmed the wide distribution of CLS in the olive growing regions of

Uruguay, and has indicated that *P. cladosporioides* is the causal agent of this disease in this country. Further research should prioritize comprehensive examination of the CLS disease cycle, including determination of the specific periods during which infections occur throughout each year. Additionally, understanding the evolution of inoculum production over time and developing a method to detect latent or asymptomatic infections would be valuable. Evaluating the effectiveness of fungicides for control of CLS and identifying the optimal timing for their application is also important. Assessing susceptibility or resistance of locally cultivated cultivars under specific environmental conditions is also likely to provide important knowledge to assist management of this disease.

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AUTHOR CONTRIBUTIONS

PL was responsible for performing the assays and data analyses, and drafted the manuscript of this papers. CL, SA and PM supervised the assays, interpretation of the results, and carried out critical revisions of the manuscript. All the authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The dataset generated during this study are available in: https://drive.google.com/drive/folders/1WAlgXhDEfFXaxZ1LHSdi2tPPt5NnAUXv

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Review

Epidemiology and control of strawberry powdery mildew: a review

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Summary. Strawberry powdery mildew, caused by *Podosphaera aphanis*, is an economically important disease for strawberry production. Typical symptoms are white mycelium on all aerial parts of affected plants, with young host tissues being the most susceptible. The pathogen overwinters on infected leaves, either as mycelium or chasmothecia, although the quantitative role of chasmothecia in epidemics are not fully understood. In spring, under favourable conditions, the fungus sporulates, disseminating conidia and causing polycyclic infections. The disease is mainly controlled using synthetic fungicides, but there is increasing interest in sustainable alternatives, including microbial biocontrol agents (e.g., *Ampelomyces quisqualis, Bacillus* spp., *Trichoderma* spp.) and substances of plant or animal origin (e.g., *Equisetum arvense*, orange oil, chitosan, whey). Physical methods, (e.g. UV-C, ozone) are also promising alternatives to fungicides. All of these strategies should be combined with appropriate agronomic practices (e.g., overhead irrigation, canopy management) to create unfavourable environments for the pathogen. However, agronomic practices have never been assessed for *P. aphanis*. Disease forecasting models and DSSs, though available, are underutilized due to their complexity and lack of validation across locations. This review presents the current state of knowledge on *P. aphanis* the available methods for control of strawberry powdery mildew, and highlights knowledge gaps relating to this host/pathogen relationship.

Keywords. *Podosphaera aphanis*, natural substances, biocontrol, agronomic practices, disease forecasting models.

INTRODUCTION

Strawberry powdery mildew (SPM), caused by *Podosphaera aphanis* (Wallr.) U. Braun and S. Takamatsu is a common disease, particularly in subtropical and tropical regions where strawberry (*Fragaria* × *ananassa* Duch) is grown (Nakzawa and Uchida, 1998; Amsalem *et al*., 2006; Gadoury *et al*., 2010; Carisse and Fall, 2021; Kasiamdari *et al*., 2021; Palmer and Holmes, 2021). Most strawberry cultivars are highly susceptible to the disease, and only very few are tolerant (Menzel, 2022). Strawberry powdery mildew is mostly managed by synthetic fungicides that are sprayed regularly from emergence of the first leaves to the end of the harvest season (Carisse *et al*., 2013a). This high use of fungicides fosters the build-up of resistant *P. aphanis* popula-

tions and has potentially negative impacts on animal and human health and the environment (Muñoz-Leoz *et al*., 2011; Rjiba-Touati *et al*., 2023). Due to increasing concerns relating to pesticides, consumers preferences have changed, and are increasingly opting for food products free of pesticide residues (Rimal *et al*., 2001). As a result, agrarian systems are moving to sustainable and eco-friendly phytosanitary solutions, which fosters research and development of innovative approaches to disease management (Deresa and Diriba, 2023).

Significant progress has been made to develop alternatives for management of SPM, and many publications confirm this strong scientific commitment. However, strawberry producers still lack effective methods for managing SPM that can be considered as viable substitutes for chemical fungicides (Deresa and Diriba, 2023).

The aim of this review is to summarize current knowledge on SPM, and to highlight gaps in understanding which, if clarified, could contribute to increased effectiveness of SPM management.

METHODOLOGY

This review is structured into the following sections: classification and morphology of *P. aphanis*, and the symptoms of SPM; epidemiology and the most significant stages of the disease cycle; conventional and alternative control methods for SPM; agronomic practices that must be integrated for effective disease control; and the most relevant predictive models, decision support systems (DSSs) and early detection systems for SPM. The review concludes by suggesting future research to improve SPM management.

The relevant literature was reviewed using Google Scholar, Scopus, and Web of Science searches, for reports published from 1962 to 2023. The following keywords were used alone and in combinations in the searches: *Ampelomyces quisqualis*, airborne inoculum, *Bacillus*, basic substances, bioassay, biochar, biological agent, biostimulants, chasmothecia, classification, cleistothecia, conidia, conidiophores, control, cultural practices, decision support system, detection, disease, distribution, environmental conditions, epidemiology, essential oils, field, fungicide, inorganic salts, irrigation, life cycle, low-toxicity compounds, machine learning, model, morphology, mycophagous mite, nutrition, overhead irrigation, overwintering inoculum, ozone treatment, plant extract, *Podosphaera aphanis,* predictive model, resistance, seaweed extract, *Sphaerotheca macularis,* symptoms, strawberry powdery mildew, *Trichoderma*, UV treatment, water stress.

The first search (46 papers) was carried out in order to select the first and the most cited records for the classification of *P. aphanis* (eight papers), its morphology (two papers), and the symptoms it causes (nine papers). A second search (27 papers) focused on the fungus life cycle (18 papers) and SPM epidemiology (11 papers). A third search (104 papers) aimed to identify the fungicides (nine papers) and the alternative products assessed for SPM control (95 papers) by focusing on classical and advanced solutions such as biological control (11 papers), inorganic salts (21 papers), plant extracts (16), seaweeds (10 papers), substances from animal origin (six papers), chitin and its derivatives (12 papers), UV-C (nine papers) and ozone technologies (six papers). The selected papers of the third search were analysed according to the research outcomes, carried out under field or laboratory conditions. When data on *P. aphanis* were lacking and alternatives for management of other powdery mildews could be useful indicators for future research, those alternatives were included in the review. A fourth search was carried out to identify agronomic practices useful for management of SPM, such as canopy management (eight papers), plant nutrition (four papers), overhead irrigation (two papers), genetic resistance (seven papers) or spray equipment (eight papers). In this fourth search, in cases where there was no literature available on SPM, literature related to other powdery mildews was analysed. The fifth search included DSSs (17 papers), and early disease detection systems (six papers). Papers were not included when they showed low quality of experimental designs and data analyses, reported low powdery mildew severity in experimental controls (only for the efficacy trials), or were redundant due to other similar and previous results.

THE PATHOGEN AND THE DISEASE

Podosphaera aphanis (*Erysiphaceae*, *Ascomycetes*) was first reported (sexual stage) in the United States of America (Geneva, New York) in 1886 (Arthur, 1886). In Europe, this fungus was identified a few years later (Salmon, 1900), when its asexual stage was also described. The causal agent of SPM was initially thought to be the same as hop powdery mildew, *Podosphaera macularis* (Wallr.) U. Braun and S. Takamatsu [formerly *Sphaerotheca macularis* (Wallr.) Magnus] (Jhooty and McKeen, 1965). In 1976, Liyanage and Royle discovered that powdery mildews of strawberry and hop were caused by two different pathogens. Recent taxonomic studies have described clear distinction between ascocarp appendages of *Podosphaera* and *Sphaerotheca* (Braun, 1982; Braun and Takamatsu 2000), which neces-

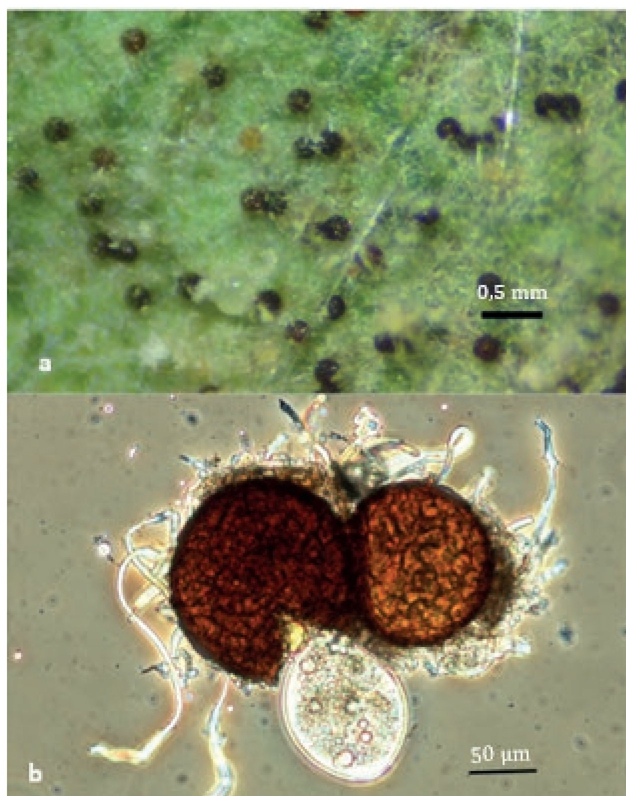

Figure 1. a) Chasmothecia of *Podosphaera aphanis* on an abaxial surface of a strawberry leaf. b) Open chasmothecium and with released ascus.

sitated a change of the genus name of the agent of SPM to *Podosphaera* (Cook *et al*., 1997; Kirk *et al*., 2001).

Morphological characteristics, originally described in 1987 (Braun, 1987), were recently displayed with digital light microscopy (Iwasaki *et al.,* 2021). The hyaline conidia of *P. aphanis* are ellipsoid–ovoid to doliiform–limoniform in shape, and contain oil and fibrosin bodies. Their dimensions are 27–33 × 18–22 µm. The appressoria, which develop on germinated conidia, are 4 µm wide. Conidiophores (dimensions 84–129 × 8–11 µm) each produce six concatenated conidia. Chasmothecia (Figure 1) are dark brown (100–125 × 65–80 µm), and are firmly attached to the surrounding mycelium. Each chasmothecium (Figure 1) contains one ascus (dimensions 60–94 × 55–76 µm), which contains eight ellipsoid to subglobose ascospores.

Symptoms of strawberry powdery mildew

The typical symptoms of SPM are white powdery patches of mycelium and conidia, spread across all aerial parts (leaves, runners, flowers, fruit) of affected host plants (Figure 2, a to i). Host tissues can be affected at all stages of development, although young organs (e.g., not fully expanded leaves, flowers, green berries) are more susceptible than older tissues (Carisse and Bouchard, 2010; Asalf *et al*., 2014). As the disease progresses, leaf edges curl upwards, and purple to reddish irregular blotches may develop on the leaf surfaces (Lambert *et al*., 2007) (Figure 2, c and d). Round black chasmothecia may be visible on abaxial leaf surfaces, in late summer/autumn (Gadoury *et al*., 2010).

Severe infections can cause strawberry yield losses of up to 30% (Carisse *et al.,* 2013b), due to the white mycelium covering ripe and unripe fruit, fruit deformation (Figure 2, g, h and i), hardening and dehydration, achene exposure (Figure 2 g), and eventual fruit decay. Beside negative impacts on fruit quality, photosynthesis reduction, plant stunting and flower abortion are also associated with SPM (Peries, 1962a; Jhooty and McKeen, 1965; Gooding *et al*., 1981; Maas, 1998; Amsalem *et al*., 2006), although no data are available on the yield losses caused by these types of symptoms.

The disease cycle of Podosphaera aphanis

The disease cycle of strawberry powdery mildew (Figure 3) has been extensively investigated. The pathogen overwinters as mycelium on living infected leaves, and sporulation recommences in spring, leading to conidium dissemination and consequent polycyclic infections (Gadoury *et al*., 2010; Iwasaki *et al*., 2021) (Figure 3). Nevertheless, *P. aphanis* can also overwinter as chasmothecia, which developed in late summer/autumn (Gadoury *et al*., 2010; Jin *et al*., 2012) on the infected host leaves, in commercial fields or in the nurseries (Peries, 1962b). In spring, commonly from early March to late May in the northern hemisphere, mature chasmothecia release ascospores, which are responsible for the early infections on plants (Gadoury *et al*., 2010) (Figure 3).

Asexual reproduction

Extensive research has been conducted on the processes of asexual reproduction during host vegetative growth, including laboratory and field studies on conidiation and polycyclic infections. These have provided insights into the dynamics of fungal development and dissemination. After infection, temperatures between 18 and 25°C at 97–100% relative humidity (RH) favour enlargement of the lesions, leading to conidiation (Miller *et al*., 2003; Amsalem *et al*., 2006). Conidiophores each develop from a generative cell that after a gradual

Figure 2. Strawberry powdery mildew symptoms. a-b) white patches on the abaxial and adaxial leaf surface, c) red blotches on the leaf surface, c-d) leaf curling, e-f) white patches on leaf and flower petioles, g) fruit deformation, h-i) white mycelium and white patches on unripe and ripe fruits.

upward elongation, produces conidium chains. Conidia are released when mature, following the individual order of development. Each time a conidium is released, the generative conidiophore cell starts to form a new conidium. The lifetime of conidiophores, from generative cell formation until the first conidium release, is approx. 125 to 150 h. At 22°C and 45–55% RH, with wind speed of 0.5 m s^{-1} (necessary for conidial detachment), each

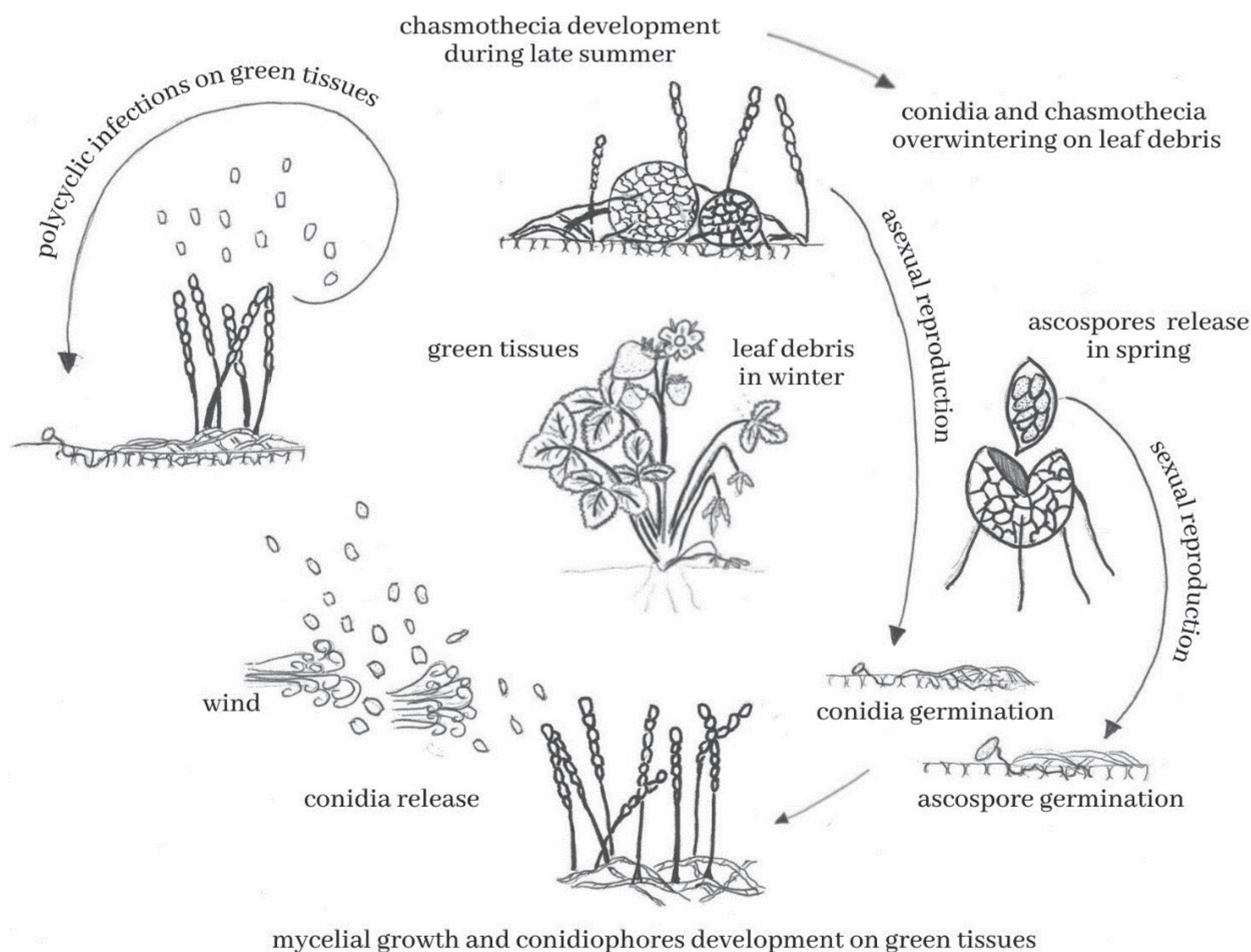

Figure 3. Disease cycle of strawberry powdery mildew.

conidiophore releases an average of 38 progeny conidia within 96 h (Iwasaki *et al.*, 2021). Within a colony lifetime (35 d after inoculation) each colony can release an average of 6.7×10^4 conidia (Ayabe *et al.,* 2022).

Under laboratory conditions (22±1°C, 45–55% RH), conidia of *P. aphanis* germinate within 4–5 h after inoculation, with each conidium forming a germ tube that develops into an appressorium (Iwasaki *et al.,* 2021). After successful host penetration, achieved by enzymatic and mechanical processes, a haustorium forms within the host epidermal cell, and typically invades the host plasma membrane 1 d after inoculation, and hyphal growth commences. Conidiophores develop 3–5 d after inoculation, and conidiation usually commences 6 d after inoculation (Peries, 1962a; Jhooty and McKeen, 1965; Iwasaki *et al.*, 2021).

Conidia can germinate between 3 and 32–38°C (Jhooty and McKeen, 1965; Sombardier *et al.*, 2009), and temperature influences the rate and speed of germination. For example, between 15 and 25°C germination of conidia varies between 85 to 88% (Amsalem *et al.,* 2006), while only 1% germination was recorded at 5 or 35°C (Amsalem *et al.,* 2006). At 5, 10, and 15°C, minima of, respectively, 25, 15 and 12 h were required for conidium germination, while between 18 and 30°C only 5 h were necessary (Peries, 1962a). Conidium germination rates are also influenced by different leaf surfaces, with is 20% greater germination on abaxial than adaxial surface (Maas, 1998; Sombardier *et al.*, 2009). As for many powdery mildews, free water is detrimental to conidia and mycelium of *P. aphanis* (Peries, 1962a; Sombardier *et al.*, 2009).

Sexual reproduction

Podosphaera aphanis is heterothallic, so initiation of chasmothecia begins when antheridium and ascogo-

nium are formed from the mycelium of different mating types. Myceloid appendages extended from the outer chasmothecia wall are directed downward to the mycelium and tenaciously attached (Asalf *et al.,* 2013). Initiation of ascocarps is regulated by temperature. The most favourable temperature for chasmothecium development is approx. 13°C, that occurs 10 to 14 days after inoculation (Asalf *et al.,* 2013). Up to 400 chasmothecia per cm^2 of leaf form after 14 d incubation at this temperature. However, chasmothecium development is largely suppressed at temperatures >13°C. For example, at 20°C the mean number of chasmothecia per cm^2 was up to 21, and the incidences of leaves bearing chasmothecia at 9 or 12°C were much greater (respectively, 92 and 93%) than at 15, or 18°C, (respectively, 7 and 6%) (Asalf *et al.*, 2013). Chasmothecia have different developmental stages: white, brown, and black when mature. Rupture of the ascus and ascospore release generally occurs within 5 min at 22 to 25°C provided that the ascocarp remains in contact with a film of water (Gadoury *et al.,* 2013).

EPIDEMIOLOGY OF STRAWBERRY POWDERY MILDEW

Environmental factors influencing the disease

Primary infections

The role and quantitative contribution of chasmothecia in initiation of SPM epidemics is not clear, and in some regions the asexual stage prevails over the chasmothecia, which are rare or absent (Howard and Albregts, 1982). This indicates a secondary role of chasmothecia in the SPM epidemiology. One possible reason is that geographically discontinuous distributions of mating types may prevent/reduce sexual reproduction (Gadoury *et al.*, 2010). A second reason could be unsuitable temperatures for the ascocarp initiation (Gadoury *et al.*, 2013). Temperature is a key environmental factor influencing ascocarp formation.

While the most favourable temperatures for the development of chasmothecia are well-documented, the conditions for chasmothecium survival during winter, and the related viability of ascospores, have been little studied. In a 4-year survey carried out in New York State and Norway, proportions of chasmothecia containing viable ascospores (i.e. positively reacting to fluorescein diacetate stain) consistently exceeded 80%. In contrast, ascospore germination on glass, investigated in the last two years of the same experiment, was highly variable, ranging from 42% to 98% (Gadoury *et al.,* 2010). This variability underscores the need to explore the factors affecting ascospore germination and infection rates. Integration of such data into powdery mildew predictive models could increase prediction precision to assist commencement of disease control treatments at the beginning of strawberry production seasons. However, quantitative estimation of initial inoculum is a challenge for all forecasting models that have been developed in other powdery pathosystems (Gubler *et al.*, 1999; Caffi *et al.,* 2011).

Secondary infections

As with the majority of *Erysiphales*, *P. aphanis* releases conidia mainly during daytime (Blanco *et al.*, 2004), and conidium release is affected by temperature and RH fluctuations. The release of conidia is directly correlated with increase in temperature and decrease in RH (Blanco *et al.,* 2004). For example, Blanco *et al.* (2004) showed, in a 2-year experiment, that in the first year minimum conidium release occurred at 12°C and 86% RH, and maximum release was at 14°C and 73% RH. In the second year release was at 13°C and 82% RH, and maximum release was at 18°C and 54% RH.

The quantity of conidia and the timing of their release are key for development of SPM epidemics (Willocquet *et al.,* 1998; Van Maanen and Xu, 2003). As for other *Erysiphales*, populations of airborne SPM conidia depend on the quantity of infected organs in a crop. For example, there is a close correlation between the weekly average aerial concentration of conidia (conidia/m^3) and the weekly average number of diseased leaves and berries (Blanco *et al.*, 2004; Van der Heyden *et al.,* 2014). As the number of conidia in the air and the infected organs in a field are closely related, the number of conidia in the air and the first visible symptoms on plants are also closely related. If promptly recognized, the critical conidium concentration threshold at which crops must be treated could be crucial to avoid field disease outbreaks. First SPM symptoms likely to become visible after 7-14 d, when the airborne conidium concentration captured with an air sampler has recorded more than 500 conidia m^{-3} d^{-1} (Carisse and Bouchard, 2010).

Because SPM is a wind-borne disease, understanding patterns of conidium dispersal under field conditions is important for implementing disease control strategies. However, accurate models of pathogen spread over time from specific inoculum sources have not been developed, although there have been attempts to measure conidium dispersal. For example, after 3 d of exposure, the dispersal radius from infected plants used as inoculum sources was 1.2-1.5 m (Peries, 1962b). The dispersal of conidia from an infected source changes according to the environment. For example, dispersal is greater under plastic

tunnels than in greenhouses (Willocquet *et al.*, 2008), and is greater in open fields than in plastic tunnels (Carisse *et al.*, 2013a). The hypothesis for these effects is that wind is less in greenhouses than in plastic tunnels, and less in tunnels than in open fields (Willocquet *et al.*, 2008; Carisse *et al.*, 2013a). In addition, this may explain why SPM dispersal in an open field is difficult to determine, because dispersal has heterogeneous patterns (Van der Heyden *et al.*, 2014).

Agronomic factors influencing the disease

Cropping systems and crop cultivars

In response to growing market demand for strawberries, cropping systems have evolved from classical field production to highly complex approaches. The need to provide high-quality product throughout the year has led to progressive replacement of short-day (June-bearing) varieties with day-neutral (or everbearing) varieties, that produce fruit for the entire season. Both varieties June-bearing and everbearing are susceptible to powdery mildew, but the latter are more exposed to pathogen infections during their life cycle in summer (Maas, 1998). Whereas June-bearing varieties produce fruit until late spring, the growing season of day-neutral varieties coincides with optimal conditions for disease development in midsummer (Maas, 1998; Blanco *et al.,* 2004; Carisse and Bouchard, 2010). Risk of infection is further enhanced where June-bearing and day-neutral varieties coexist. There is an overlapping production at the beginning of the season of new transplanted day-neutral plants with overwintering infected June-bearing varieties, which are sources of inoculum (Fall and Carisse, 2022). In subtropical regions, to ensure high yields, June-bearing varieties are planted in mid-summer for the first harvest, in late summer, and, after overwintering in the field, these crops each produce a second harvest in the following late-spring. The day-neutral varieties, on the other hand, are planted in mid-spring for a single growing season, that partially overlaps with the growing cycle of the June-bearing plants (Carisse and Fall, 2021).

As well as high susceptibility of everbearing strawberry varieties, the adoption of soilless production on raised beds in plastic tunnels or greenhouses gives environmental conditions that are conducive to powdery mildew (Xiao *et al.*, 2001). Under coverage, SPM is not inhibited by rain and/or prolonged leaf wetness, which can stop conidium germination and eventually kill conidia in open fields. In addition, polyethylene/glass shading decreases sunlight intensity, favouring powdery mildew development because these pathogens are strongly photosensitive (Amsalem *et al.*, 2006; Elad *et al.*, 2007).

Plant water stress

Effects of plant water stress on *P. aphanis* infections has not been extensively studied, although for other powdery mildews host water stress reduces hyphal growth, slowing colonisation of new tissues, and also disrupts conidiation (Ayres and Woolacott, 1980; Caesar and Clerk, 1985). Xu *et al.* (2013) and Rossi *et al* (2020) showed positive correlations among plant hydration, disease susceptibility, and pathogen fitness. For example, 21 d after inoculation, water stressed plants showed slight reductions in disease severity on abaxial leaf surfaces compared to the well-hydrated plants (Rossi *et al.,* 2020). Host water stress also affects conidium germination. Germination rates differed for conidia collected from plants grown at different soil moisture levels. Germination, assessed on water agar, increased linearly from 0 to 30% for conidia collected from plants grown in soil moisture levels ranging from 0 to approx. 53%.

CONTROL METHODS AND APPROACHES

Fungicides

In the European Union, synthetic fungicides authorised for the control of SPM and categorised based on their modes of action (Frac Code List, 2022) (Table 1), belong to the following groups: hydroxy-(2-amino-) pyrimidines (A2), succinate dehydrogenase inhibitors (C2), quinone outside inhibitors (QoIs; C3), (C5), demethylation inhibitors (G1), and others with unknown modes of action (U). To reduce risks of selecting resistant pathogen populations, fungicides with different modes of action must be combined in appropriate disease management strategies (Palmer and Holmes, 2021). The majority of active substances authorised for the use against *P. aphanis* belong to few mode of action groups, resulting in recurrent use of a limited number of products with the same modes of action. Some of these fungicides, such as the triazoles (demethylation inhibitors) have favoured emergence of resistant *P. aphanis* populations (Sombardier *et al.*, 2010; Palmer and Holmes, 2021).

Among the authorised active ingredients, only sulphur has multisite mode of action, which can be used to mitigate emergence of fungicide resistant *P. aphanis* populations (Peres and Mertely, 1969). However, although sulphur has low mammalian toxicity and has a long use history, including in organic farming, this

Table 1. Fungicides authorised for use against strawberry powdery mildew in at least one European Union Member State (EU pesticide database, April 2023). The active ingredients are grouped according to the FRAC Code list, 2022.

Active substance	Target Code	Group name
Bupirimate	A2	Hydroxy- (2-amino-) pyrimidines
Boscalid	C2	Succinatedehydrogenase inhibitors
Cyflufenamid	U6	Phenylacetamides
Difenoconazole	G1	Demethylation inhibitors
Fluopyram	C2	Succinatedehydrogenase inhibitors
Fluxapyroxad	C2	Succinatedehydrogenase inhibitor
Meptyldinocap	C5	Uncouplers of oxidative phosphorylation
Penconazole	G1	Demethylation inhibitors
Pyraclostrobin	C3	Quinone outside inhibitors
Tetraconazole	G1	Demethylation inhibitors
Trifloxystrobin	C3	Quinone outside inhibitors
Sulphur	M02	Inorganic

chemical can negatively impact beneficial arthropods (Beers *et al.*, 2009).

In addition to selection of resistant pathogen populations, some compounds, such as the triazoles (demethylation inhibitors; G1), are posing risks for animals and humans (Muñoz-Leoz *et al.*, 2011; Rjiba-Touati *et al.*, 2023). Also because of slow environmental degradation of these chemicals (EFSA, 2010), they have been associated with detrimental human health consequences, including infertility and disruptions in neurobehavioural functioning (Menegola *et al.*, 2006; Zhang *et al.*, 2016).

Bioprotection

There has been increased research to develop alternatives to synthesized fungicides. However, intrinsic bias often occurs, with tendency to publish positive results while ignoring the negative outcomes. This leaves an important gap in understanding of effectiveness of these alternatives, which may result increased expectation for efficacious products. When applied in the field, these products fail to control target diseases, either due to overestimation of efficacy or to lack of knowledge of factors that may reduce their effects, such as optimal concentrations, and timing and frequency of applications. This underlines the importance considering negative results which could contribute realistic evaluations. There are also discrepancies between published research and results obtained by the industry. While a range of commercial products have been officially authorised for the use in SPM control (and are therefore of proven efficacy), this often lacks robust confirmation in the scientific literature. Industry operators may not disclose efficacy data, which hinders the advancement of research efforts. This lack of information impedes collective progress in SPM control and raises concerns about the "robustness" of the effectiveness of these active substances.

Several categories of alternatives to fungicides have been defined. Although their analysis is beyond the scope of the present review, the suggestion of Stenberg *et al.* (2021) is relevant: *"bioprotection can be used as an excellent umbrella term that encompasses protection provided by either living agents or non-living substances of biological origin [...] with low impact on human health and the environment'*. In the present review we divide the alternatives into groups based on their nature and/or origins.

Inorganic salts

Several inorganic salts have been tested for efficacy in suppressing fungal pathogens, but when considering powdery mildews, potassium and sodium bicarbonates (Homma *et al.*, 1981; Crisp *et al.*, 2006a), potassium silicate (Menzies *et al.*, 2019) and potassium phosphate (Reuveni *et al.,* 1995; Reuveni and Reuveni, 1998) have been the most investigated. For SPM, there are fewer reports, and only potassium and sodium bicarbonates and silicates have been sufficiently assessed. For example, potassium and sodium bicarbonates (4 g L^{-1}) showed promising efficacy, in leaf bioassays, for control of *P. aphanis*, with, respectively, 87% and 84% reductions in hyphal biomass (Pertot *et al.,* 2007). The promising laboratory results were not fully confirmed in the field, where prolonged applications of a combination of potassium bicarbonate and potassium silicate at 6 g L^{-1} gave 85% disease incidence compared to 88% for untreated controls (Gomez *et al.,* 2017). A much greater rate of potassium bicarbonate (20 g L^{-1}), if integrated with fungicides, has given promising outcomes. Two applications of potassium bicarbonate were as effective as the systemic fungicide myclobutanil, suggesting a potential role for potassium bicarbonate in integrated disease management (Dodgson, 2007). Comparative studies assessing the effectiveness of inorganic salts versus conventional fungicides or their combinations, could improve their application strategies. Even if mode of action has yet to be fully defined (Deliopoulos *et al.*, 2010), activity of bicarbonates likely occurs when the salts come into contact with the pathogen. This interaction inhibits sporulation and fungal development due to detrimental osmotic effects of K^+ imbalance, spore dehydration and increases

in leaf surface pH (Ziv and Zitter, 1992; Kettlewell *et al.*, 2000; Pertot *et al.*, 2007). Bicarbonates need frequent applications to be effective, as is emphasized in label guidelines of commercial products (e.g., Karma 85, Certis Europe), that suggest 7-10 d interval between treatments. However, these repetitive treatments can possibly cause residual deposits and phytotoxicity.

Potassium silicate is another inorganic salt that has been extensively tested against SPM. Silicon (Si) is associated with beneficial effects on mechanical and physiological characteristics of plants, depending on whether it is applied to roots or to canopies. For example, potassium silicate 100 mg L^{-1} applied once to strawberry roots in hydroponics decreased disease severity by 17% (Kanto *et al.*, 2004). This compound at 500 mg L^{-1}, applied at an average of 0.86 g m^{-2} d^{-1} during cultivation (Kanto *et al.*, 2006) also suppressed SPM in soil by up to 15%. Kanto *et al.* (2007) demonstrated that hydroponic Si fertilization decreased disease severity and reduced fungal fitness. This was shown by reductions in germination of conidia collected from Si-treated plants compared to the controls. Germination rates were 49.7% for Si-treated plants and 67.2% for the controls. This protective role of root Si fertilization was attributed to Si accumulation in leaves, which hinders cuticle penetration by pathogens (Seal *et al.*, 2018). This theory was supported by identification of Si transporters in strawberries, providing genetic evidence that strawberry is receptive for Si fertilisation (Ouellette *et al.*, 2017). However, silicic acid is the only soluble form that plants can absorb to successfully store Si in leaves and decrease disease severity (Ouellette *et al.*, 2017). Under a daily potassium silicate fertilization (1.7 mM Si) leaf accumulation can reach up to 3% Si on a dry weight basis (Ouellette *et al.*, 2017). Silicon is also as an elicitor of plant resistance, and induces several defence-related reactions, such as the over-production of enzymes (e.g., polyphenoloxidase and peroxidase) and antifungal compounds (e.g., flavonoids and phytoalexins) (Wang *et al.*, 2017).

Elicitation of resistance to SPM in strawberry has yet to be assessed. Although potassium silicate is promising when applied to plant roots, when applied to leaves this compound was less effective (Palmer *et al.*, 2006; Jin, 2015; Gomez *et al.*, 2017). Root applications influence various aspects of plant physiology and defence mechanisms, which may have greater disease suppression effects than foliar applications. The mode of action of foliar-applied potassium silicate for reducing powdery mildew has not been determined, but formation of physical barriers and osmotic effects on leaf surfaces may contribute to disease suppression (Bowen *et al.*, 1992; Rodrigues *et al.*, 2009).

Plant extracts

Plant extracts are complex mixtures containing bioactive compounds, that are obtained by physical processes such as distillation and extractions of/from leaves, stems, of fruit (SANCO, 2012). In the EU, plant extracts used as plant protection products are authorised according to Regulation (EC) No 1107/2009 (EU, 2009). Several plant extracts have shown promising results for suppressing powdery mildews, with various mechanisms including inhibition of spore germination and mycelium growth, and disrupting fungal reproductive structures (Marei *et al.*, 2012; Silva *et al.*, 2020). Among plant extracts, essential oils and aqueous extracts are promising groups for disease management.

Essential oils are concentrated hydrophobic liquids extracted from plants, by distillation with water or steam, mechanical processes (e.g., pressing or grinding), or dry distillation (ISO 9235, 2021). These oils contain volatile compounds (ISO 9235, 2021) that have diverse biological activities, including antifungal properties (Cavanagh, 2007; Ferraz *et al.*, 2022). Among these, oils from *Thymus* spp. L., *Mentha* spp. L., *Melaleuca alternifolia* (Maiden and Betche) Cheel (tea tree oil) and *Citrus sinensis* (L.) Osbeck (orange oil) have been tested against several powdery mildew species (Reuveni *et al.*, 2020; Mostafa *et al.*, 2021; Frem *et al.*, 2022), but limited information is available for SPM.

Orange oil is the only essential oil authorized in the EU and in at least one European Union Member State for control of SPM (European Pesticide Database, 2023). However, only a few papers report efficacy of orange oil against SPM (Prodorutti *et al.*, 2019). For example, orange oil, if applied weekly, was as effective against SPM as most conventional fungicides. Disease severity was reduced from 81% (untreated control) to 14% by penconazole and 19% by orange oil (Prodorutti *et al.*, 2019). The active component of orange oil is limonene, a volatile compound that disrupts fungal cell membranes and inhibits spore germination (Marei *et al.*, 2012; Silva *et al.*, 2020). Beside antifungal properties, orange oil has insecticidal properties, as is common with essential oils in general (Isman, 2020), possibly affecting beneficial insects and disrupting ecosystem balance. This highlights the importance of holistic pest management strategies that target pathogens but also do not generally impact the field environment.

Aqueous plant extracts may include secondary metabolites, phenolic compounds and enzymes that can directly affect pathogen physiology and growth (Tavares *et al.*, 2021). For example, *Equisetum arvense* L., and *Salix* spp. L. cortex extracts can control some powdery

mildew species, although with slight efficacy (Marchand *et al.,* 2014; Frem *et al.,* 2022). Among plant aqueous extracts, those from *E. arvense* are authorised in the EU as basic substances and in at least one European Union Member State for control of SPM (EU Pesticide Database, 2023). Although the mode of action is unknown, silicon as a major component of *E. arvense* reduces effects of excessive moisture on leaves and inhibits fungal growth. This involves creation of physical barriers of Si on leaf surfaces combined with osmotic effects that absorb excessive moisture favouring fungal proliferation (Bowen *et al.*, 1992; Rodrigues *et al.,* 2009).

Seaweed extracts

The first report of seaweed against powdery mildews was that of Stephenson (1966), and research on these extracts has expanded (Li *et al.*, 2020; Elagamey *et al.*, 2023). Abundant and common brown seaweeds such as *Ascophyllum nodosum* (L.) Le Jolis, *Ecklonia maxima* (Osbeck) Papenfuss and *Laminaria digitata* (Hudson) J.V. Lamouroux, are the most frequently used for their plant growth promoting activities (Khan *et al.*, 2009). In the EU, most seaweed extracts used in agriculture are considered as fertilizers (EU, 2019). However, seaweeds have also been acknowledged as potential alternatives for plant protection products, due to their capacity to enhance plant disease resistance by interacting with secondary metabolism and defence-related processes (EIBC, 2012; OECD, 2017). For example, laminarin, a storage glucan extracted from *L. digitata*, is an authorised active substance for SPM in the EU, with demonstrated positive results in laboratory and field tests. In leaf assays, laminarin decreased *P. aphanis* conidium germination by 75% (Bajpai *et al.,* 2019), while in greenhouse tests laminarin with a reduced chemical dosage, gave 1.7% SPM infestation, which was similar to the complete chemical scheme (Melis *et al.*, 2017).

The laminarin mode of action against SPM not been investigated. However, its plant protection activity has been studied for several plant species and involves several key elements. The compound elicits production of defence compounds, such as phytoalexins (Aziz *et al.,* 2003), and synthesis of pathogenesis-related proteins (Tziros *et al.,* 2021). It may also directly interact with the pathogen, reducing conidium germination and fungal growth (Hu *et al.,* 2012; de Borba *et al.*, 2022).

Substances from animal origins

Cow's milk and whey have been studied for their plant growth-promoting activity (Sharratt *et al.,* 1959; Ahmed Hashim, 2019), and as alternatives to synthetic fungicides. Fresh cow's milk, at concentrations greater than 10% in water, applied twice a week, was as effective (10% severity) as fenarimol and benomyl (9%) for reduce powdery mildew of zucchini squash, compared to the water control (56% severity), after 1 month since treatment (Bettiol, 1999). Similarly, 10% whey applied twice a week powdery mildew severity (caused by *Podosphaera xanthii* (Castagne) U. Braun and Shishkoff) by 71-94% in cucumber and 81-90% in zucchini, compared to experimental controls (Bettiol *et al.*, 2008). Cow's milk and whey are already authorized in the EU as basic substances and in at least one European Union Member State for use against several powdery mildews (EU Pesticide Database, 2023).

Effects of milk and whey against powdery mildews may involve more than one mode of action. Electron spin resonance and scanning electron microscopy showed that fresh milk and whey applied to grape leaves infected by *Erysiphe necator* Schwein. led to the collapse of fungal hyphae and conidia within 24 h after treatments, likely because of release of free radicals, fatty acids, and lactoferrin by the milk microbial community (Crisp *et al.*, 2006b). Despite high efficacy, the European Food Safety Authority has raised concerns about potential food allergies associated with lactose and milk proteins derived from the use of whey for plant protection (SANTE, 2021). Consequently, its application is restricted in the EU only to approved crops during plant growth stages devoid of fruit (EU Pesticides Database, 2023). Without additional safety data, milk/whey for SPM control could be authorized only in the EU at the beginning of crop growth, when disease outbreaks are commonly rare, making the alternative of little use for growers.

Chitin and chitin derivatives

Chitin, an amino polysaccharide, is a structural supporting components of fungal cell walls, and insect, nematode, and crustacean exoskeletons (Latgè, 2007). Chitin and chitin oligosaccharides have been assessed as plant protection agents (Li *et al.*, 2020), because they are environmentally friendly and highly degradable (Yeul and Rayalu, 2013). These compounds have antimicrobial activities and elicit host defence mechanisms. When recognized by plant cells, they trigger several immune responses (Xing *et al.*, 2015; Li *et al.*, 2020), including lignification and cytoplasmic acidification (Barber *et al.,* 1989).

Chitosan, the N-deacetylated derivative of chitin, is the most extensively studied among chitin fragments. Chitosan is a family of molecules with different sizes and compositions, so it has ductile chemical and physi-

cal properties (Aranaz *et al*., 2021). Chitosan stimulates plant defences and growth (Chakraborty *et al*., 2020), but also has filmogenic and fungicide properties against spore and mycelium growth (Martínez-Camacho *et al*., 2010; Meng *et al*., 2010). Chitosan is effective against several powdery mildew pathogens. *Sphaerotheca fuliginea* (Schltdl.) Pollacci on cucumber cotyledons in Petri dishes was inhibited by one preventive treatment of 2.5% chitosan (Moret and Muñoz, 2009). Similarly, a weekly foliar treatment of 0.5% chitosan on cutting roses decreased infections by *Podosphaera pannosa* (Wallr.) de Bary (Wulf *et al*., 2023). However, field studies with chitosan suggest it should be applied when pathogen levels are low (Wulf *et al*., 2023). Although chitosan has been authorised in the EU as a basic substance and in at least one European Union member state for SPM control, there are no reports of efficacy in scientific literature.

Chitosan fragments known as chitooligosaccharides (COS) have been tested in combination with pectin (oligogalacturonides, OGA) as elicitors of plant resistance in a formulation referred to as COS-OGA (Ferrari *et al*., 2013). Because of proven efficacy (van Aubel *et al*., 2014), COS-OGA has been authorised in the EU for the use against several powdery mildews, including SPM. However, no efficacy data are available for chitosan against SPM.

Microbial biocontrol agents

Microbial biocontrol agents (BCAs) are microorganisms that act against phytopathogens with various mechanisms (e.g., competition for resources, antibiosis, hyperparasitism, and induced resistance), and can control plant diseases (Köhl, *et al*., 2019). Several BCAs with different modes of action have been studied against SPM: *Ampelomyces quisqualis* Ces., *T. harzianum* Rifai, and *Bacillus* spp. Cohn are the most investigated. *Ampelomyces quisqualis* is a hyperparasite of several powdery mildew fungi (Sundheim, 1982; Falk *et al*., 1995). *Trichoderma* spp. strains are mycoparasites that can produce antifungal metabolites, and can induce host resistance (Vinale *et al*., 2008). *Bacillus* spp. produce many antimicrobial compounds and can induce resistance on plants (Pérez-García *et al*., 2011). The microbes commonly have good efficacy when applied under controlled laboratory/greenhouse conditions, but their efficacy decreases under commercial field conditions. For example, *in vitro*, *A. quisqualis* AQ10 and *T. harzianum* T39 decreased SPM hyphal biomass by, respectively, 46 and 74%, compared to untreated controls, but these organisms were not as effective as *B. amyloliquefaciens* (formerly *B. subtilis)* QST 713 Cohn that achieved results that were similar to those from to chemical pesticides (99% inhibition of hyphal biomass) (Pertot *et al*., 2007). However, under field conditions, the exclusive use of these microorganisms throughout crop growing seasons without integrating fungicides has been proven insufficient. Contrary to bioassay results, *T. harzianum* T39, in an integrated programme, had the greater activity than *A. quisqualis*. The average fruit incidence in the two locations was 25% for *T. harzianum* and 44% for *A. quisqualis* (Pertot *et al*., 2008). In contrast, their efficacy against leaf severity was variable across locations. Currently, *A. quisqualis* AQ10 and *B. amyloliquefaciens* QST 713 are authorized in the EU and in at least one European Union Member State for SPM control (EU Pesticide Database, 2023).

Inhibition of SPM conidiation (80.7% reduction) on leaf discs was also obtained combining *B. subtilis* ABiTEP GmbH FZB24 and *Metarhizium anisopliae* (Metschn.) Sorokīn (Sylla *et al*., 2013). However, no studies have reported assessments under field conditions. *Bacillus pumilus* Meyer and Gottheil QST2808 is also authorized in the EU and in at least one European Union Member State for SPM control (EU Pesticide Database, 2023). This microorganism, under field conditions, demonstrated high consistency against SPM compared to other tested BCAs. It showed better efficacy compared to a 14 d fungicide application regime, but not in comparison with a 7 d fungicide application schedule (Berrie and Xu, 2021). No data are available for efficacy of *B. pumilus* QST 2808 against *P. aphanis* under controlled conditions.

Understanding the epidemiology of SPM disease and the environmental conditions for survival and/or optimal growth of BCAs in the field are considered key factors for successful control strategies (Pertot *et al*., 2008). Variability in BCA efficacy under field conditions often stems from misuse of these living organisms, treating them as if they were synthetic fungicides, so use of BCAs is more complex than for chemical agents (Legein *et al*., 2020). Applying BCAs at specific stages of the pathogen cycle could be more strategic than frequent treatments during crop growth seasons, when environmental conditions may not be favourable for BCA growth. For example, *A. quisqualis* AQ10, when applied at the end of a crop growth season under suitable temperature and RH conditions can reduce inoculum for the following growing season. Ensuring BCA efficacy also includes assessing compatibility with conventional fungicides when developing integrated pest management programmes. For example, *A. quisqualis* is incompatible with commonly used chemicals against SPM, including penconazole, pyrimethanil, tebuconazole, cyprodinil, fosetyl-aluminium, azoxystrobin, and metalaxyl (Roberti

et al., 2002). Research on biocontrol agents has a long history, but there has been little recent research focusing on SPM. The research community may have recognized that the previous approaches are not productive for addressing this issue.

Fungivorous biocontrol agents

While microbial BCAs have predominantly dominated biocontrol efforts against powdery mildews, fungivorous insect biocontrol agents, have recently emerged as potential contenders (IBMA, 2022). Pijnakker *et al*, (2022) reported that the mycophagous mite *Pronematus ubiquitus* McGregor gave promising results against tomato powdery mildew (*Oidium neolycopersici* L. Kiss), by decreasing disease severity to 4%, compared to 32% for untreated controls, 8 weeks after mite release. The mites were in greater numbers where powdery mildew was severe. In addition, Pijnakker *et al.* (2022) suggested that for effective disease control this mite must be released preventatively. For SPM control, *P. ubiquitous* has not been assessed scientifically, but is currently being investigated by the industry, and is at first stages of market development (IBMA, 2022). Although the precise contribution of conidium nutrition and plant-mediated effects on powdery mildew resistance, remain unclear, there is potential for determining these interactions. It is also important to develop understanding of whether *P. ubiquitus* is present in each territory of investigation, as potential field releases of alien mites may not be permitted (Heimpel and Cock, 2018).

Other control means

Crop canopy management

Plant canopies have important roles in the development of powdery mildew diseases, which are favoured by host vigour and high plant density in many host species (Jarvis *et al.,* 2002). Dense canopies create microclimates (i.e., high humidity, low ventilation, low light penetration) that favour pathogen growth (Aust and Hoyningen-Huene, 1986; Keller *et al*., 2003), and suitable canopy management can reduce infection risks. Direct effects of canopy management on SPM control have not been validated in robust research. However, some studies indicate positive correlations between SPM severity and canopy density. For example, breeding for SPM resistance is leading to the selection of cultivars with reduced canopy densities due to consistent genetic correlations observed between host susceptibility and high canopy density (Kennedy *et al*., 2014). Although research on SPM is lacking, studies on other powdery mildews suggest practices that can be also tested for strawberry. For powdery mildew of hop (*P. macularis*) removal of highly susceptible climbing shoots and reductions in canopy density improved disease management and fungicide distribution (Gent *et al*., 2012; Gent *et al*., 2016). In grapevine, vertical trellis system and spring pruning reduced powdery mildew by up to 32% (Austin and Wilcox, 2011). Canopy thinning in strawberry crops has been assessed for yield optimization (Sønsteby *et al*., 2021), but has not been comprehensively investigated for SPM management. Similarly, removal of highly susceptible strawberry runners could reduce risks of SPM (Eccel *et al*., 2010), but this is yet to be precisely quantified.

Host nutrition

Balanced mineral nutrition is important for plant self defense, and when specific elements are either deficient or over-abundant, plants can become vulnerable to particular pathogens (Huber and Haneklaus, 2007). High nitrogen inputs have been associated with increased risk of fungal diseases. For SPM under experimental conditions, Xu *et al.* (2013) reported a 54% increase of nitrogen above fertigation standard (from 128 to 197 mg L^{-1}) applied from the beginning of bloom resulted in an 8% increase in disease severity. For deficiencies in the other macro- and micro-elements, there are no published reports relating to SPM susceptibility.

Some soil amendments may enhance plant defence against biotic stresses. For example, biochar can induce plant resistance by improving chemical and physical soil properties (e.g., water holding capacity, nutrient availability, soil texture), and by enhancing soil microbial activity such as plant growth promoting rhizobacteria (Schmidt *et al*., 2021). For strawberry, incorporation of 3% biochar into potting mixture resulted in high expression of defence-related genes and a related decrease of SMP (Harel *et al*., 2012).

Overhead irrigation

Although *P. aphanis* develops well under high RH (Amsalem *et al*., 2006), free water prevents conidium germination (Peries, 1962a). Water sprays on plant canopies can control SPM but could also promote pathogenic fungi that are favoured by a wet canopy, such as *Botrytis cinerea* Pers. and *Colletotrichum* spp. Corda. However, since micro-sprinklers are commonly used to spray water

to reduce high temperature stress during summer (Liu *et al.,* 2021), well-balanced overhead irrigation can be used to reduce SPM. For example, application of pulsed water mist has shown promising results: applications of 660 mL min^{-1} for 1 min four times a day was as effective as standard fungicide treatments, in high tunnel and open field conditions (Asalf *et al.*, 2021). Overhead irrigation also reduces SPM severity when applied for long periods. For example, after 67 d of mist treatments, severity of powdery mildew decreased from 80 to 17% in high tunnels and from 73 to 22% in the open field, compared to untreated controls. Application of pulsed misting for 1 min four times a day did not increase in *B. cinerea* infections, indicating that if water was correctly applied, grey mould could be reduced (Asalf *et al.,* 2021). Although overhead irrigation reduced the disease, procedures (i.e., frequency, volume, application methods) were not fully explored for maximizing efficacy.

Fungicide spray equipment

Spray equipment can also affect pest control (Ebert and Downer, 2006), and this is particularly the case for SPM because applied fungicide must reach the undersides of leaves, lower leaves and the fruit. This is particularly difficult when strawberry plants develop dense canopies. Low technology devices (i.e. hand-held and cannon sprayers) may not provide adequate and even fungicide distribution (Balsari *et al*., 2008; Bondesan *et al*., 2015). These devices are widely used in strawberry high-tunnels in Mediterranean regions (e.g., Italy and Spain) (Sánchez-Hermosilla *et al*., 2012; Cerruto *et al.,* 2018), because they are inexpensive and easily adaptable to horticultural crops. Cannon sprayers also distribute plant protection agents at high pressure (>20 bar) and rates (1500–2500 L ha^{-1}), producing spray drift that can contaminate soil and may increase operator exposure (Sánchez-Hermosilla *et al*., 2011, 2012; Cerruto *et al*., 2018). In technologically advanced greenhouses, sprayers with increased efficiency, such as vertical booms (Braekman *et al*., 2010), or autonomous pesticide spraying robots (Abanay *et al*., 2022), have been associated with improved better canopy coverage and reduced application volumes compared with cannon and hand-held sprayers (Braekman *et al.,* 2010).

Ultraviolet light

Light is an important factor for minimising fungal development and stress responses in plants, and ultraviolet light (UV) can suppress powdery mildews in several crop plant hosts (Gadoury *et al*., 1992; Suthaparan *et al*., 2012; Pate *et al*., 2020). For strawberry, the application of UV once or twice per week during night-time (60 s followed by 4 h dark period) resulted in up to 90% reduction of SPM incidence and severity compared to the controls (Janisiewicz *et al*., 2016). However, UV-based methodology is still at early commercial development, and has various challenges. For example, UV technology is not adaptable to diverse rural growing systems, such as high-tunnels and open fields. In some cases, machines may not be able to access tunnels and/or move between benchtop rows. Application parameters (UV dose, light exposure durations, treatment frequencies) are not yet optimised and standardised. A range of doses spanning from 30 to 200 J m^2, administered once or twice per week, or at 10 d intervals, have been assessed (Van Delm *et al*., 2014; Janisiewicz *et al*., 2016; Suthaparan *et al*., 2012; Ledermann *et al*., 2021), without determining the best application schedule. Antifungal effects also only occur only irradiated host surfaces and UV light poorly penetrates crop canopies, and uniform light distribution is difficult in multi-layered crop canopies, giving limited SPM control on abaxial leaf surfaces (Delorme *et al*., 2020). Implementing UV light technologies is expensive: beside the initial costs that include purchase of UV equipment, installation, and the necessary modifications to the existing infrastructure, there are extra costs for electricity and frequent replacement of UV lamps (Rea *et al.,* 2022). For these reasons, growers must carry out careful cost/benefit analyses when evaluating the feasibility of UV light for SPM control.

Ozone

Ozone (O_3) has antimicrobial activity and is rapidly decomposed in the environment. In the food industry O_3 is used to safely disinfect food, and as postharvest treatments to increase shelf-life of fruit and vegetables (Tzortzakis and Chrysargyris, 2017). For plant protection, O_3 has been tested against powdery mildews of several horticultural crops under controlled conditions, both as fumigant and as ozonated water (Hibben and Taylor, 1975; Rusch and Laurence, 1993; Khan and Khan, 1999; Fujiwara and Fujii, 2002; He *et al*., 2015). Effects of O_3 on powdery mildews and plants depends on concentration: at too low levels powdery mildews may be not harmed, while at too high levels host phytotoxicity may occur. For example, increasing concentrations of gaseous O_3 (from 50 to 200 ppb) applied intermittently (7 h d^{-1} for 7 d) on cucumber plants in closed-top chambers, decreased powdery mildew colonization from 70 to 23%. In addition, 50 ppb of O_3 increased the germination conidia collect-

ed from treated plants, while conidia exposed to greater concentrations (100 and 200 ppb) were smaller and had reduced germination compared to untreated controls. High O_3 concentrations (i.e., 200 ppb) can cause foliar necroses (Khan and Khan, 1999). Ozonated water gives similar results (Fujiwara and Fujii, 2002). Although some growers currently use ozonated water, its efficacy against SPM has not yet been assessed (Fujiwara and Fujii, 2004). Devices to spray ozonated water are available (e.g., MM-Biozono, MMSpray, Italy; Mowat, Gr Gamberini, Italy), and are also tailored for strawberry production (e.g., GZO-D, ZonoSistem, Spain), but no definitive data are available (e.g. minimum exposure times, effective dosage) (Fujiwara and Fujii, 2004).

Genetic resistance to strawberry powdery mildew

Breeding for resistant varieties is an effective disease management strategy, provided that plants bear high-quality fruit, are well-suited to local cultivation regions and have adequate and long-lasting tolerance or resistance to pathogens. Resistance in strawberry to *P. aphanis* has low durability and is variable under different environmental conditions (Menzel, 2022). Whether this behaviour is related to unstable resistance genes or different virulence of SPM strains is unknown (Nelson *et al.,* 1995). Several genes may control levels of infection, and under natural conditions inoculum density varies leading to differential elicitation of systemic resistance (Kennedy *et al*., 2013). In a plant breeding programme, beside inoculum level, other variables (e.g., climatic conditions, growing systems, time of season) may influence strawberry responses to pathogens, making comparison of results obtained in different breeding programmes challenging. Defining the optimum breeding methodology and conditions for development of resistant strawberry cultivars could be helpful (Menzel, 2022). Marker-assisted selection can accelerate cultivar improvement, but SPM resistance in strawberry is probably regulated by complex genetics with several additive genes involved. To date, several genes have been associated with SPM resistance (Menzel, 2022), including nine QTL genes (Cockerton *et al*., 2018; Sargent *et al*., 2019), seven TGA genes (Feng *et al*., 2020a) and 68 MLO sequences (Tapia *et al*., 2021).

Predictive models and Decision Support Systems

Reductions of fungicide use can also be achieved by optimizing, and thus reducing, numbers of spray applications, and predictive models and Decision Support Systems (DSSs) can help growers identify optimum timing of pesticide applications. Predictive models are based on empirical data collected from the field and/or under controlled conditions, and forecast disease development (Van Maanen and Xu, 2003). DSSs are interactive computer-based systems, which use predictive models, data analysis techniques, and recommend/support actions for farmers to manage diseases (Sprague and Carlson, 1982). Both of these tools are useful to schedule fungicide treatments, thereby avoiding unnecessary applications (Lázaro *et al*., 2021). For SPM, several predictive models (Carisse *et al*., 2013a, 2013b) and DSSs (Table 2) have been developed (Gubler *et al*., 1999; Eccel *et al*., 2010; Bardet and Vibert, 2011; Dodgson *et al*., 2021; Carisse and Fall, 2021; Fall and Carisse, 2022). However, the developed models, excepting that of Gubler *et al.* (1999), have not been validated in different locations. This decreases the reliability of the models, as agricultural conditions can vary widely from one region to another. Without validation it is therefore difficult to assess model robustness and accuracy in different environmental contexts.

Predictive models

Several models, as mentioned in the epidemiological section of this review, have been developed in Canada. For example, Carisse *et al.* (2013a) characterized a close relationship between SPM incidence and severity to define an economic loss threshold for fungicide interventions. In another model, Carisse *et al.* (2013b) described a strong positive linear relationship between seasonal crop losses, disease severity and daily mean airborne conidium concentration, to potentially define a severity and airborne conidium concentration threshold for fungicide interventions. Carisse and Bouchard (2010) defined windows of high leaf and berry susceptibility for June-bearing and everbearing strawberry cultivars.

DSS developed by Carisse and Fall

From these models, Carisse and Fall in 2021, modelled a DSS based on a decision tree forecast (the outcome of several algorithms that offered a model, following a subset of classification rules visualised and exemplified as a tree) (De Ville, 2013). This model forecasts risk of infection, firstly from airborne inoculum concentration and number of susceptible leaves, and then using mean RH, mean daily number of hours at temperature between 18 and 30°C, and mean daily number of hours at saturation vapour pressure between 10 and 25 mmHg

Table 2. Decision support systems developed for management of strawberry powdery mildew.

Reference	Aim	Input drivers	Output	Validation	Treatment reduction	Commercial application
Dodgson *et al.* (2021)	Disease development forecast based on the number of hours with favourable conditions	T°, RH%	Daily risk predicted on cumulative h of conducive conditions, recommendation of action	2009-2020, under tunnels, UK	30% fungicide reduction	Agri-tech
Bardet and Vibert (2011)	Disease development forecast based on favourable conditions for fungal stages	T°, RH% and rainfall	Graphical representation of disease progression and infection risk in 4 d period	2006-2007 under tunnels and glasshouse, 2010 under tunnels, France	Not available	Inoki
Eccel *et al.* (2010)	Disease development forecast based on weather data, growing system, agronomic practices, host susceptibility.	T°, RH%, daily disease incidence, type of sprayer, tunnel height, overhead irrigation, cultivar susceptibility, time of disease onset, time since last treatment, presence of runners	Daily risk of disease outbreak and risk forecast in the next 3 d, recommendation of action	2007 under tunnels, Italy	60% fungicide reduction	Not available
Hoffman and Gubler (2002)	Ascosporic infection and disease development forecast based on whether data	T° and leaf wetness	Treatment interval threshold according to risk index	2002 in open field, California, in 2008 in open field, Quebec	40% fungicide reduction in California, 0% in Quebec	Not available
Fall and Carisse (2022)	Dynamic simulation of inoculum load and fungal development based on weather data	T°, rainfall, RH%, plant density, initial airborne inoculum concentration	Daily SPM severity, warning and action threshold and related crop loss	2006, 2007, 2008, 2015, 2016 and 2018 in raised beds open field, Quebec	Not available	Not available
Carisse and Fall (2021)	Decision tree forecast of infection based on weather data	Airborne inoculum concentration, susceptible leaves, RH%, T°, vapour pressure	Daily infection risk, warning	2015, 2016, 2018 in raised beds open field, Quebec	Not available	Not available

during the previous 6 d. Carisse and Fall (2021) noted that the main characteristic of their prediction system was understanding that groups of variables can affect SPM e development, and that different combinations of these variables can result in similar disease severities. For example, low inoculum amounts and a limited number of susceptible leaves, but conducive weather, may yield the similar severities as scenarios of high inoculum, few susceptible leaves and less favourable weather conditions. The factor potentially hindering use of the model could be detection of airborne conidium concentrations, that Carisse and Fall (2021) suggested analysing manually, twice weekly using microscopy, for each strawberry field.

DSS developed by Fall and Carisse

Fall and Carisse (2022) developed a DSS according to a dynamic simulation model, which simulates the asexual life cycle of *P. aphanis* and its related severity. This model considers at which rate *P. aphanis* changes growth stage with time, according to weather conditions, simulating daily conidium production and resulting disease severity. In the model *P. aphanis* stages (initial inoculum, conidium germination dropout population, germinated conidia, cumulative proportion of diseased leaf area, secondary inoculum) are regulated and influenced by rate variables, such as sporulation rate,

germination rate, lesion increase rate (defined by algebraic equations), that in turn are influenced by intermediate variables such as daily temperature, rainfall, RH and the number of leaves per plant (35,000 plant ha^{-1} in a 0.91 m row spacing field), estimated on a daily basis. The model is based on evidence that in May at least one lesion m^{-2} of strawberry field is sporulating, and the initial inoculum load in 1 ha of strawberry is assumed to be 1,000,000 conidia (Blanco *et al.,* 2004; Carisse *et al.*, 2013a, 2013b). According to the initial inoculum value, the model starts running each day, estimating inoculum load based on weather data and fungal development, thus predicting powdery mildew severity and related crop loss. According to disease severity, warning and action thresholds are simulated on a daily basis. For cost-effective management of SPM, crop managers in Quebec may tolerate 1% yield losses (warning) but not more than 5% losses (action).

UC Davis DSS

In California, a DSS developed by UC Davis (Gubler *et al*., 1999) was another attempt to forecast SPM epidemics, assessing risks and action thresholds. This model was developed for grape powdery mildew (caused by *E. necator*) and then applied for SPM (Hoffman and Gubler, 2002). The model focuses on forecasting ascospore infection to refine fungicide application timing at the start of each cropping season (Gubler *et al*., 1999; Hoffman and Gubler, 2002). This model assesses ascospore release according to leaf wetness and temperature, considering that at least 12–15 h of continuous leaf wetness at 10-15°C average temperatures are necessary for release. After the ascospore infection occurs, the model changes into the risk assessment phase, relying solely on temperature impacts on pathogen reproductive rate. To start an epidemic, the pathogen requires three consecutive days with at least 6 h between 21 and 30°C. If these conditions are not met, the index resets to zero; otherwise, the model initiates estimation of an infection index (from 0 to 100). Thresholds of action and frequency of intervention depends on risk. If the risk index remains low (<30), interval between treatments decreases (between 14 and 21 d). If risk index is increases (>60), shorter intervals between applications are recommended (maximum, 7 d interval). The model was validated in 2002 under open field conditions, reducing 40% of fungicide treatments, compared to a calendar-based programme. However, after several tests in Quebec, the model did not accurately predict SPM at the beginning of the season, probably due to the wide range of favouring conditions, This resulted in similar numbers of fungicide applications prescribed as for calendar-based schedules (Bouchard, 2008).

Safeberry DSS

In Italy, Eccel *et al.* (2010) modelled the SafeBerry DSS, based on forecasted daytime temperatures over 3 d, and risk factors including daily disease incidence in a tunnel, type of sprayer, tunnel height, overhead irrigation, cultivar susceptibility, time of disease onset, time since last treatment, and presence of runners. Suitability of weather conditions for disease development was categorised according to day-time temperature as follows: low suitability (≤18 or >26°C), medium suitability (18 < T° ≤ 20 or 25 < T° ≤ 26°C), and high suitability (20 < T° ≤ 25°C). Outputs of this model are daily assessment of disease outbreak risk at the daily time/temperature during the previous 6 d and forecasted in the next 3 d, the favourability of temperature for disease in the next 3 d, and then a recommendation for action. The model includes two action possibilities: either 'Do not spray today' or 'Apply as soon as possible'. In the second scenario, a selection of recommended fungicides is provided, based on their modes of action, risks for pathogen resistance development, and timing restrictions prior to harvest. With this system in 2007, under tunnel conditions, up to 60% reductions in fungicide treatments were obtained.

DSS developed by Bardet and Vibert

In France, Bardet and Vibert (2011) developed a DSS that modelled five stages of the *P. aphanis* life cycle (inoculum dispersal, infection, mycelium growth, sporulation, and disease progression), as influenced by meteorological variables of temperature, RH, and precipitation. The model was based on evidence that conidium germination occurs between 5 and 32°C, mycelium growth is interrupted above 35°C, sporulation occurs between 7 and 28°C. and conidium dispersal occurs in low humidity conditions. The maximum threshold accepted by the model is set at RH <65% over a minimum duration of 8 h. At RH >85% for at least 5 h, germination occurs rapidly. The index risk separately considers the conditions favourable for infection, mycelium establishment with sporulation, and lesion development, and each stage has a 0 to 5 value. For example, 5 is assigned to infection under favourable conditions for the fungus (temperature between 20 and 26°C, and 85% < RH < 99%). For strawberry cultivars that are particularly susceptible, the model allows additional risk to be

set. The model gives graphical representation of periods suitable for pathogen dissemination, infection and mycelium growth for a 4 d period. Fungicide treatments can appear on the graph provided their application dates and effectiveness duration are entered. The model was validated with experiments conducted in 2006 and 2007 under tunnel and glasshouse conditions, and in 2010 under tunnel conditions. This DSS is available for growers through the web platform Inoki (Ctifl, 2023).

Strawberry Powdery Mildew Forecasting Model DSS

In the United Kingdom, a DSS implementing 15 years of historical data was developed by Dodgson *et al.*, (2021). The model is based on laboratory and field evidence that *P. aphanis*, under optimum conditions (>15.5 and <30°C, 60% RH), takes 144 h (disease conducive hours) to complete a cycle from conidium germination (6 h) to growth of elongating secondary hyphae and sporulation (138 h). The system then extends according to weather conditions (15.5°C, the minimum temperature for spore germination; 18°C, the minimum temperature for sporulation, at 60% RH). According to sensitivity analysis under field conditions, temperature is the main factor influencing fungal development and sporulation. Other secondary weather parameters with lower impacts on the prediction system, such as leaf wetness, were removed to simplify the rules of the forecast. Once one cycle is completed, a daily risk is predicted and used for guiding fungicide applications. The prediction system uses a 'traffic light' colour scheme indication to represent the progression of accumulated hours of conducive conditions. When 125 accumulated hours are reached, the line changes from yellow to red, indicating high risk of conidium production. A fungicide should normally be applied before the elapsed time reaches 144 accumulated hours, to prevent *P. aphanis* sporulation. When a fungicide application is made, the growers record this manually in the software, and reset the system to zero and the process repeats. Unlike the Carisse and Fall (2021) decision tree, the Dodgson *et al.* (2021) model does not essentially require accurate estimation of susceptible leaves and airborne inoculum, and these variables are deemed to be limiting. Instead, the Dodgson *et al.* (2021) forecast always assumes a standard presence of inoculum, and susceptibility for all crops. To effectively manage powdery mildew, growers are required to start each growing season with a *clean-up spray* treatment, as this was also confirmed by greater infection in crop where clean-up spray was neglected. Relying on these assumptions and the risk forecast, control of powdery mildew was demonstrated with 30% fungicide reduction. The model has been validated from 2009 to 2020 under tunnel conditions. To date, the Dodgson *et al.* (2021) online real-time web-based prediction system is used and sold with commercial licencing (Strawberry Powdery Mildew Forecasting Model, Agri-tech Service, United Kingdom).

New tools for early detection

Early disease detection is often complex and time-consuming, and for SPM, prompt recognition of the disease in the field is difficult (Carisse *et al.*, 2013a). However, rapid development of advanced agricultural technologies, such as machine learning and vision, has helped capture of disease images, and, therefore, detection of pathogen presence and abundance in the field (Liu and Wang, 2021). Machine vision-based recognition may replace traditional naked eye identification with computing science. Robust models have recently been developed to detect SPM on strawberry leaves with high accuracy (>94%) (Shin *et al.*, 2020, 2021).

At research level, analyses of volatile organic compounds released by diseased crops is another potential machine learning technique for disease detection. These compounds are potential biomarkers for warning and forecasting disease spreading in fields (Li *et al.*, 2019). The approach is based on plant emission of unique profiles of Volatile Organic Compounds (VOCs) when attacked by a pathogen, which differ from profiles from undamaged plants, allowing interactive signalling with neighbouring plants and release of danger signals. Nearby undamaged plants may recognise this novel profile and activate physiological changes that enhance their readiness to future pathogen attacks (Effah *et al.*, 2019). For powdery mildew detection, this has only been studied for *B. graminis*, where sensitivity and specificity of six wheat VOCs have been identified as possible biomarkers for disease detection (Hamow *et al.*, 2021). SPM identification and detection by VOC analyses has not been assessed, although greenhouse-grown strawberry plants could be excellent candidates for VOC analyses.

CONCLUSIONS

This review has critically considered the extensive research on SPM, attempting to identify knowledge gaps that warrant further investigation. Given the similarities of SPM with the other powdery mildews, the available data on other species could be used to inspire future research. In addition, factors related to growers' approaches to plant protection strategies could be

considered. For example, natural substances are used as supplementary and marginal tools in disease management spray programmes, which are still largely based on synthetic fungicides. To overcome this problem, data on natural substance efficacy under various environmental conditions should be generated and made available in the public domain. Exploring new more effective application methods may also increase farmer confidence on alternative products. Natural substances and antagonistic microorganism often have limited field persistence, and frequent and/or appropriate timing of applications are required. Exploration of solid set spraying systems, especially in greenhouses and tunnels, could provide valuable new direction for SPM management. Assessment of the impacts of agronomic practices on SPM, and validation of SPM forecasting models across diverse strawberry-producing regions also deserve research effort.

Genomic, transcriptomic, and metabolomic technologies could provide powerful tools for development of innovative plant protection strategies. Although promising, biotechnological tools remain underexplored for SPM control. These technologies could be useful for assisting the breeding for resistant host varieties. For example, naturally occurring or experimentally induced inactivation and/or mutation of MLO genes (e.g., by gene silencing and genome editing) may provide strong and long-lasting immunity/resistance to the fungus (Wan *et al*., 2020).

Transcriptomics and metabolomics can offer unique approaches for identifying host resistance traits (Castro-Moretti *et al*., 2020). When transcriptomic information is coupled with metabolomic analyses, plant defence mechanisms can be better understood (Wink, 1988), and this knowledge could guide targeted interventions. For example, metabolomics can guide selection or breeding of plant cultivars with increased levels of defence molecules. For example, SPM infections influence strawberry plant metabolism (Duan *et al*., 2022): alongside phenols, ten chitinases are upregulated in infected plants, indicating the role of chitinase in reaction to *P. aphanis* (Duan *et al*., 2022). For example, determining substances that can mimic pathogen effects on strawberry chitinase overexpression, or identifying cultivars that are can further overexpress these enzymes, could result in new tools for disease management, as has been demonstrated by some reported attempts (Feng *et al*., 2020b; Zhang *et al*., 2021; Yin *et al*., 2022). Alongside overexpression of plant defence related pathways, gene silencing with expression of RNAi constructs against host and/or pathogen target genes could be assessed (Capriotti *et al*., 2020). Through the utilization of host and/or pathogen RNA interference (RNAi), specific pathogen genes could be silenced by degrading their messenger RNAs. This process can hinder translation of the RNA into proteins, thereby disrupting pathogen ability to carry out normal biological processes (Zotti *et al*., 2018). RNAi-based fungicides are at early stages of development, but they have already been assessed against grape powdery mildew (*E. necator*), giving up to 64% reduction in conidium production compared to experimental controls (McRae *et al*., 2023).

These new biotechnologies, although powerful, may be of limited use due to high costs (both for research and implementation), and because of existing restrictive regulations. Therefore, innovative investments and policy interventions are necessary to guarantee sufficient knowledge advancements from research on SPM.

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Research Papers

Identification of pathogens causing brown rot of stone fruit in Cuneo province (Italy) and assessment of sensitivity to azoxystrobin, cyprodinil, fenhexamid, fludioxonil, and tebuconazole

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Summary. *Monilinia* spp. cause brown rot and blossom blight of stone fruit. This study characterized the diversity of *Monilinia* spp. associated with stone fruit rots in the Cuneo province, the major fruit production area in Piedmont, and assessed their sensitivity to azoxystrobin, cyprodinil, fenhexamid, fludioxonil and tebuconazole. Species diversity was determined by PCR amplification and sequencing of isolate internal transcribed spacer (ITS) regions. Sensitivity to fungicides was determined by measuring *in vitro* mycelium growth on fungicide-amended media. Fifty isolates were obtained from apricot, cherry, or peach fruits with typical brown rot symptoms. Thirteen isolates were identified as *M. fructicola*, and 37 as *M. laxa*. Nine isolates of *Monilinia laxa* and two of *M. fructicola* had resistance factor (RF) values greater than 10 for different fungicides. The greatest (RF) value (48.96) was measured for azoxystrobin against the *M. fructicola* isolate CVG 1514. Among the *M. laxa* isolates, isolate CVG 1547 had the greatest RF value to cyprodinil, while isolate CVG 1709 had RF values greater than 10 for cyprodinil and tebuconazole. A systematic and wider sampling should be carried out in the Piedmont region to determine the distribution of fungicide resistant *Monilinia* spp. in stone fruit crops. The use of site-specific fungicides remains the most effective strategy for control brown rot, and continued monitoring for fungicide resistance within *Monilinia* spp. populations is recommended.

Keywords. *Monilinia*, fungus characterization, chemical control.

INTRODUCTION

Monilinia fructicola, M. laxa, M. fructigena and *M. polystroma* are the causal agents of blossom blight and brown rot of stone fruit (peach, nectarine, plum, apricot and cherry) (Holb, 2008; Chen *et al.*, 2013; Abate *et al.*, 2018). Chemical control of these diseases is the most effective strategy to reduce pathogen inoculum and disease incidence (Mustafa *et al.*, 2021). Site-specific fungicides are currently available against brown rot in Europe (Commission Implementing Regulation (EU), No. 540/2011), where one to three spray applications are applied from flowering to ripening stages. Frequent use of site-specific fungicides increases the risk of selection of fungicide resistant pathogen populations, reducing fungicide effectiveness and disease control.

In Italy, brown rot is the most important fungal disease of stone fruit, both in orchards and post-harvest storage. Before 2008, *M. laxa* and *M. fructigena* were the only recorded brown rot pathogens (Pratella, 1996). In 2008, Pellegrino *et al.* (2009) reported *M. fructicola* in Cuneo province (Piedmont) for the first time, which was included in the EPPO A2 list (no. 153, OEPP/EPPO, 1997) as a quarantine pest. Later, *M. fructicola* was reported in additional Italian regions (Martinelli *et al.*, 2013; Landi *et al.*, 2016; Martini *et al.*, 2016; Montuschi *et al.*, 2016; Abate *et al.*, 2018), while in 2014, *M. polystroma* was also first reported in Italy, causing brown rot of peach (Martini *et al.*, 2014).

Demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors (SDHIs), amino acids and protein synthesis inhibitors and signal transduction inhibitors fungicides are classified according to the Fungicide Resistance Action Committee (FRAC, 2022). In Italy, site-specific fungicides used in stone fruit orchards against brown rot include anilinopyrimidines (e.g. cyprodinil, pyrimethanil), phenylpyrroles (e.g. fludioxonil), triazoles within the DMIs (e.g. tebuconazole, penconazole), SDHIs (e.g. boscalid, penthiopyrad), hydroxyanilides (e.g. fenhexamid), and QoIs (e.g. azoxystrobin, pyraclostrobin, trifloxystrobin). Postharvest fungicide applications are not approved for stone fruit in Italy.

The FRAC considers the three main species of *Monilinia* as pathogens of moderate risk for development of fungicide resistance, as resistant isolates have been reported both under field and laboratory conditions (FRAC, 2020). Reductions in sensitivity of *Monilinia* spp. to QoIs has been reported in Brazil (May-De Mio *et al.*, 2011; Pereira *et al.*, 2017) and in the United States of America (Holb and Schnabel, 2007; Amiri *et al.*, 2010). Isolates resistant to DMIs were reported in North America (Schnabel *et al.*, 2004; Chen *et al.*, 2013) and South America (Lichtemberg *et al.*, 2016; Pereira *et al.*, 2020). In Europe, reductions in sensitivity of *Monilinia* spp. to several fungicide classes, including dicarboximides, DMIs and hydroxyanilides, have been reported in Spain (Egüen *et al.*, 2015), Italy (Bustos Lòpez *et al.*, 2012), Greece (Malandrakis *et al.*, 2013) and Serbia (Hrustić *et al.*, 2018).

Some information is available on *Monilinia* spp. distribution in Italy, but little is known about the fungicide sensitivity of these pathogens. (Abate *et al.*, 2018; Mancini *et al.*, 2021). For this reason, a survey was carried out in Piedmont to obtain isolates associated with affected fruit, to characterize pathogen species diversity and determine their sensitivity to fungicides. This study aimed to: a) monitor presence and species of *Monilinia* spp. associated with brown rot in stone fruit orchards in the Cuneo province, the major Piedmont stone fruit production area; and b) determine sensitivity of obtained *Monilinia* isolates to azoxystrobin, tebuconazole, fenhexamid, cyprodinil and fludioxonil.

MATERIALS AND METHODS

Field survey, sampling and fungus isolations

In June and July 2021, samples were collected from commercial stone fruit orchards (cherry, peach and apricot; Table 1). Single sampled orchards, representative of a small subset of this stone fruit production area, were situated in four towns in the Cuneo province. Isolations were carried out from brown rot affected fruit of different cultivars (Table 1). Portions (5–8 mm) of each symptomatic fruit were surface sterilized with 1% sodium hypochlorite for 30 sec, then rinsed in sterile distilled water for 1 min, and dried on sterile absorbent paper. Small fragments (2–3 mm) were cut from lesion margins and plated on potato dextrose agar (PDA, Merck) amended with 25 ppm of streptomycin sulphate (PDA-S). The plates were incubated at 25±1°C, and after 48 to 72 h incubation, single hyphal tips from margin of resulting colonies were cut and placed individually on PDA plates to establish pure cultures. The obtained isolates were used for determinations of *in vitro* sensitivity to fungicides and molecular identification (Table 1). Stock cultures of isolates are kept at -80°C in the University of Torino (Italy) culture collection.

DNA extraction, PCR amplification and sequencing

Mycelium was scraped from surfaces of 10-d-old cultures grown on PDA, and placed into 2 mL capacity

Table 1. Isolate details and GenBank accession numbers for isolates included in this study.

Species	Isolate	Host	Cultivar	Origin	GenBank No. ITS
Monilinia laxa	CVG 1506	Cherry	Sweetheart® Sumtare	Manta, Cuneo, Italy	OP317580
	CVG 1507	Cherry	Sweetheart® Sumtare	Manta, Cuneo, Italy	OP317581
	CVG 1508	Cherry	M2029	Manta, Cuneo, Italy	OP317582
	CVG 1509	Cherry	Kordia	Manta, Cuneo, Italy	OP317583
	CVG 1513	Cherry	Selah®	Manta, Cuneo, Italy	OP317586
	CVG 1535	Cherry	Giant Red	Manta, Cuneo, Italy	OP317588
	CVG 1536	Apricot	Tom Cot	Manta, Cuneo, Italy	OP317589
	CVG 1540	Cherry	Sweetheart® Sumtare	Manta, Cuneo, Italy	OP317593
	CVG 1541	Cherry	Coralise	Manta, Cuneo, Italy	OP317594
	CVG 1542	Cherry	Coralise	Manta, Cuneo, Italy	OP317595
	CVG 1543	Cherry	M2003	Manta, Cuneo, Italy	OP317596
	CVG 1544	Cherry	M2003	Manta, Cuneo, Italy	OP317597
	CVG 1566	Cherry	Giant Red	Manta, Cuneo, Italy	OP317602
	CVG 1567	Cherry	Sweet Saretta	Dronero, Cuneo, Italy	OP317603
	CVG 1568	Cherry	Sweet Saretta	Dronero, Cuneo, Italy	OP317604
	CVG 1569	Cherry	Sweet Saretta	Dronero, Cuneo, Italy	OP317605
	CVG 1633	Peach		Falicetto, Cuneo, Italy	OP317606
	CVG 1642	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317608
	CVG 1643	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317609
	CVG 1644	Peach		Falicetto, Cuneo, Italy	OP317610
	CVG 1645	Peach		Falicetto, Cuneo, Italy	OP317611
	CVG 1648	Peach		Falicetto, Cuneo, Italy	OP317613
	CVG 1650	Peach		Falicetto, Cuneo, Italy	OP317615
	CVG 1692	Peach		Falicetto, Cuneo, Italy	OP317616
	CVG 1693	Peach		Falicetto, Cuneo, Italy	OP317617
	CVG 1699	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317618
	CVG 1702	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317620
	CVG 1703	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317621
	CVG 1705	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317622
	CVG 1707	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317623
	CVG 1709	Peach		Manta, Cuneo, Italy	OP317624
	CVG 1712	Peach		Manta, Cuneo, Italy	OP317625
	CVG 1713	Peach		Manta, Cuneo, Italy	OP317626
	CVG 1714	Peach		Manta, Cuneo, Italy	OP317627
	CVG 1715	Peach	Nettarina W3	S. Pietro del Gallo, Cuneo, Italy	OP317619
	CVG 1716	Peach		Manta, Cuneo, Italy	OP317628
	CVG 1717	Peach	N/A	Manta, Cuneo, Italy	OP317629
M. laxa	CBS 298.31	N/A	N/A	Ireland	HQ856917
	BPZK	Peach	N/A	Serbia	KC544793
	MDA12	N/A	N/A	United States	HQ846948
Monilinia fructicola	CVG 1510	Cherry	Kordia	Manta, Cuneo, Italy	OP317584
	CVG 1511	Cherry	Kordia	Manta, Cuneo, Italy	OP317585
	CVG 1514	Cherry	Selah®	Manta, Cuneo, Italy	OP317587
	CVG 1537	Cherry	M2043	Manta, Cuneo, Italy	OP317590
	CVG 1538	Cherry	M2043	Manta, Cuneo, Italy	OP317591
	CVG 1539	Cherry	M2043	Manta, Cuneo, Italy	OP317592
	CVG 1545	Cherry	M2003	Manta, Cuneo, Italy	OP317598
	CVG 1546	Cherry	M2003	Manta, Cuneo, Italy	OP317599

(Continued)

Table 1. (Continued).

Species	Isolate	Host	Cultivar	Origin	GenBank No. ITS
	CVG 1547	Cherry	Selah®	Manta, Cuneo, Italy	OP317600
	CVG 1563	Cherry	M2043	Manta, Cuneo, Italy	OP317601
	CVG 1635	Peach		Falicetto, Cuneo, Italy	OP317607
	CVG 1647	Peach		Falicetto, Cuneo, Italy	OP317612
	CVG 1649	Peach		Falicetto, Cuneo, Italy	OP317614
M. fructicola	Ft	N/A	N/A	France	HQ846967
	XP1	Peach	N/A	Chaoyang, Beijing	KR778937
	P169	Nectarine	N/A	Italy	FJ411109
Monilinia fructigena	CBS 101500	N/A	N/A	Poland	KR778933
	CBS 101499	N/A	N/A	Spain	KR778932
	SPBA	Plum	N/A	Serbia	KC544805
Monilinia polystroma	CBS 102686	N/A	N/A	Japan	HQ846944
	HML-3	Plum	N/A	China	GU067539
	09-G4	Apricot	N/A	Switzerland	JN128835
Monilia yunnanensis	GP18	Peach	N/A	Yanqing, Beijing	HQ856917
Botrytis cinerea	BCE4	Tomato	N/A	Beijing	HQ856917

centrifuge tubes. Total DNA was extracted from all isolates using the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek), following the manufacturer's instructions. Species identification was achieved by DNA amplification and sequencing of the nuclear ribosomal internal transcribed spacer (ITS) regions of the isolates. For each isolate, ITS was amplified using universal primers ITS1 and ITS4 (White *et al.*, 1990). Reactions were each carried out using a Taq DNA polymerase kit (Qiagen), in a final volume 25 µL, containing 2.5 µL of Qiagen PCR buffer 10×, 1.4 µL of 25mM $MgCl_2$, 0.5 µL of each dNTP (10µM), 0.5 µL of each primer (10µM), 0.2 µL of Taq DNA polymerase, and 25 ng of DNA. Amplification was carried out using the following conditions: initial preheating for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min and with a final extension at 72°C for 7 min. An aliquot (5 µL) of PCR product for each reaction was separated by electrophoresis at 100V in a 1% agarose gel (VWR Life Science AMRESCO® Biochemicals), and then stained with GelRed™ in 1× Tris-acetate-EDTA (TAE) buffer (40 mM Tris acetate and 1 mM EDTA, pH 8.0). PCR products were sequenced in forward directions by Eurofins Genomics Service. Obtained sequences were analyzed using Geneious v. 11.1.5 software (Auckland, New Zealand), and were blasted against the NCBI's GenBank nucleotide database to determine the closest relatives of the studied isolates. Isolate sequences, including sequences downloaded from GenBank, were initially aligned with the software MAFFT v. 7 online server (Katoh and Standley, 2013), and were then manually adjusted in MEGA v.7 (Kumar *et al.*, 2016). Phylogenic analysis, based on Maximum Parsimony (MP), was carried out using Phylogenetic Analysis Using Parsimony (PAUP) v. 4.0b10 (Swofford, 2003).

Fungicides and in vitro *sensitivity of isolates*

Commercial formulations of azoxystrobin (Ortiva®, 250 g L^{-1} active ingredient (a.i.), Syngenta), cyprodinil (Chorus® 50%. a.i., Syngenta), fenhexamid (Teldor Plus®, 500 g L^{-1} a.i. Bayer CropScience), fludioxonil (Geoxe® 50% a.i., Syngenta) and tebuconazole (Folicur® WG 25% a.i., Bayer CropScience) were used in this study. These fungicides were each dissolved in sterilized water, and stock solutions were prepared and stored at 4°C. Sensitivity of fungal isolates was assessed at concentrations of 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, or 300 µg mL^1 for each fungicide. Autoclaved agar medium was cooled to 50°C, and 10 mL of fungicide-amended medium was dispensed in each Petri plates. Sensitivities to azoxystrobin, fenhexamid, fludioxonil and tebuconazole were assessed on PDA. Salicylhydroxamic acid (SHAM; Sigma-Aldrich) was added to azoxystrobin amended PDA at 100 µg mL^{-1} to prevent test fungi from commencing alternative respiration. To determine isolate sensitivity to an anilinopyrimidine fungicide, as reported by Myresiotis *et al.* (2007), cyprodinil was added to minimal medium containing (per liter) 10 g glucose, 1.5 g K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)2SO_4$, 0.5 g $MgSO_4.7H_2O$, and 12.5

Table 2. Concentrations and resistance factors (RF) of azoxystrobin, cyprodinil, fenhexamid, fludioxonil and tebuconazole at which mycelium growth of *Monilinia laxa* and *Monilinia fructicola* were inhibited by 50% (EC_{50} values).

Species	Parameter	*Monilinia laxa*	*Monilinia fructicola*
Azoxystronbin	EC_{50} (μg mL^{-1}) max/min	3.44/0.05	7.47/0.03
	$EC_{50\ mean}$	0.37	1.19
	RF max/min	22.54/0.35	48.96/0.19
Cyprodinil	EC_{50} (μg mL^{-1}) max/min	1.11/0.03	0.29/0.06
	$EC_{50\ mean}$	0.18	0.12
	RF max/min	25.33/0.62	6.59/1.32
Fenhexamid	EC_{50} (μg mL^{-1}) max/min	0.49/0.04	1.09/0.12
	$EC_{50\ mean}$	0.14	0.28
	RF max/min	4.53/0.41	9.97/1.13
Fludioxonil	EC_{50} (μg mL^{-1}) max/min	0.12/0.02	0.19/0.03
	$EC_{50\ mean}$	0.05	0.07
	RF max/min	4.13/0.81	6.30/0.87
Tebuconazole	EC_{50} (μg mL^{-1}) max/min	0.42/0.02	0.37/0.13
	$EC_{50\ mean}$	0.18	0.22
	RF max/min	11.43/0.63	9.95/3.61

g agar. PDA mycelial plugs were taken from margins of 10-d-old colonies with using a cork borer (0.6 cm diam.). The plugs were then each placed upside down at the centres (one plug per plate) of Petri plates (9 mm diam.) containing PDA + fungicide. Unamended PDA plates were used as experimental controls. The plates were then incubated at 25°C for 10 d in the dark. Each isolate was tested in triplicate for each fungicide and concentration. Mean colony diameters (minus the diameter of the inoculation plugs) were determined by measuring two diameters at right angles to each other in each plate, at after 7 and 10 d incubation. These data were expressed as daily mycelium growth rates and percentages of growth inhibition relative to the unamended controls.

Data analyses

The fungicide EC_{50} values (concentrations inhibiting mycelium growth to 50% of experimental controls) were determined by regressing percentages of relative mycelium growth inhibition against the $\log_{10}$ of fungicide concentrations. EC_{50} values for each isolate were calculated with the GraphPadPrism® software (version 9.1.1), using the log dose-response relation. EC_{50}s allowed calculation of Resistance Factors (RFs), which showed sensitivity levels of the different isolates (Schnabel *et al.*, 2004). Each RF is defined as the EC_{50} of the isolate divided by the mean EC_{50} value of sensitive isolates. As sensitive/standard reference isolates were not available for *Monilinia* spp. for the selected fungicides, and a baseline population was also not available, we defined sensitive/susceptible isolates based on Minimum Inhibitory Concentration (MIC) for each fungicide. MIC measures sensitivity to antifungal agents (Xie *et al.*, 2012), and is defined as the lowest concentration of fungicide that completely inhibits fungal growth. Average EC_{50} of sensitive isolates for each fungicide, defined as isolates for which growth was completely inhibited at the MIC concentration, were used to calculate RF values, and isolates were classified as resistant to each active ingredient when the RF was greater than 10 (Campia *et al.*, 2017).

RESULTS

Sampling, isolation and molecular identification of Monilinia *isolates*

A total of 50 isolates were obtained from peach, cherry or apricot fruits with typical brown rot symptoms. The ITS phylogeny consisted of 63 sequences, including *Botrytis cinerea* (BCE4) as outgroup. A total of 437 characters were included in the phylogenetic analysis, 20 characters were parsimony-informative, 6 were variable and parsimony-uninformative, and 411 were constant. A maximum of 1,000 equally MP trees were saved (Tree length = 32, CI = 0.875, RI = 0.969, RC = 0.848). Bootstrap support values from the MP analysis are included in Figure 1. The phylogenetic tree showed that the isolates clustered in two different lineages, with reference isolates of *M. fructicola* and *M. laxa*. Thirteen isolates (26% of the isolates collected) clustered with *M. fructicola*, and 37 (74%) clustered with *M. laxa*. *Monilinia fructicola* was identified from only two orchards: in Manta (Cuneo) with ten isolates and in Falicetto (Cuneo) with three isolates. *Monilinia laxa* was predominantly collected from cherry and peach fruits, while only one isolate (CVG 1536) was isolated from apricot. *Monilinia fructicola* was isolated only from cherry and peach fruits. None of the collected isolates were identified as *M. fructigena* or *M. polystroma*.

In vitro *sensitivity to fungicides*

The fungicides used for *in vitro* sensitivity tests inhibited growth of *M. laxa* and *M. fructicola* isolates at different levels. EC_{50} values for each fungicide for the fifty isolates were obtained from *in vitro* assays for mycelial growth (Figure 2).

Four *M. fructicola* isolates were resistant to azoxystrobin, with EC_{50} values, respectively, of 7.47, 3.44, 2.82

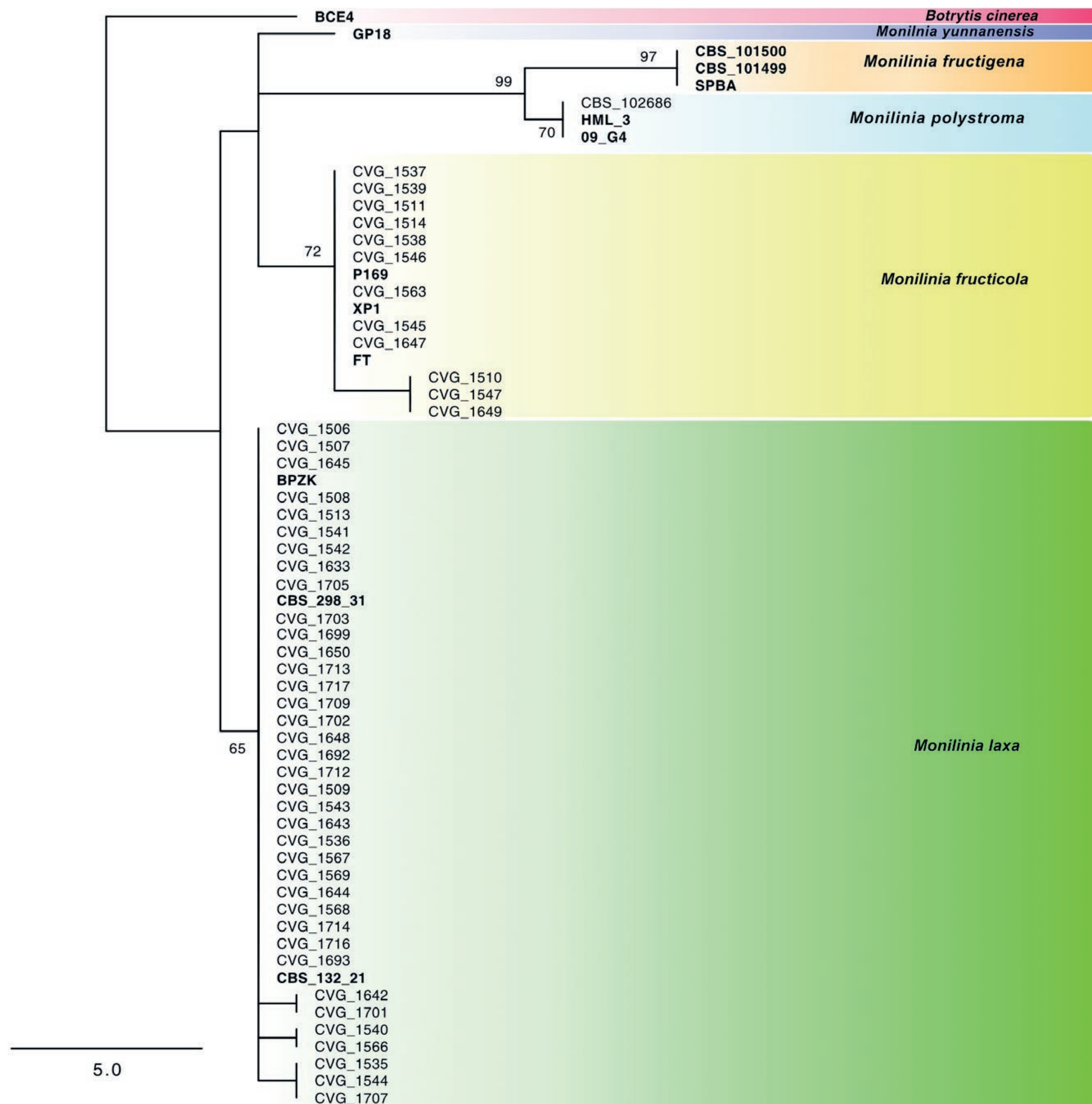

Figure 1. The most parsimonious tree obtained from a heuristic search of ITS sequence alignments of *Monilinia* spp. Bootstrap support values are shown at the nodes. The scale bar represents the number of changes. The tree was rooted to *Botrytits cinerea* (BCE4). GenBank isolates are indicated in bold font.

and 2.37 μg mL^{-1}, giving resistance factor (RF) values of, respectively, 48.96, 22.54, 18.48 and 15.52. Within the *M. laxa* isolates, CVG 1643 had the greatest sensitivity to azoxystrobin (EC_{50} = 0.05 μg mL^{-1}), and lowest sensitivity was recorded for CVG 1540 (EC_{50} = 3.44 μg mL^{-1}). Resistance factor values for azoxystrobin in *M. fructicola* isolates ranged from 48.96 to 0.19. The minimum inhibitory concentration (MIC) for azoxystrobin of sensitive isolates was 3 μg mL^{-1}, while some isolates grew at concentrations up to 100 μg mL^{-1}. Two isolates of *M. laxa* (CVG 1540, CVG 1566) and two isolates of *M. fructicola* (CVG 1514, CVG 1547) had RF values greater than 10.

For cyprodinil, one *M. laxa* isolate (CVG 1703) was resistant to the fungicide, with a high EC_{50} of 1.11 μg

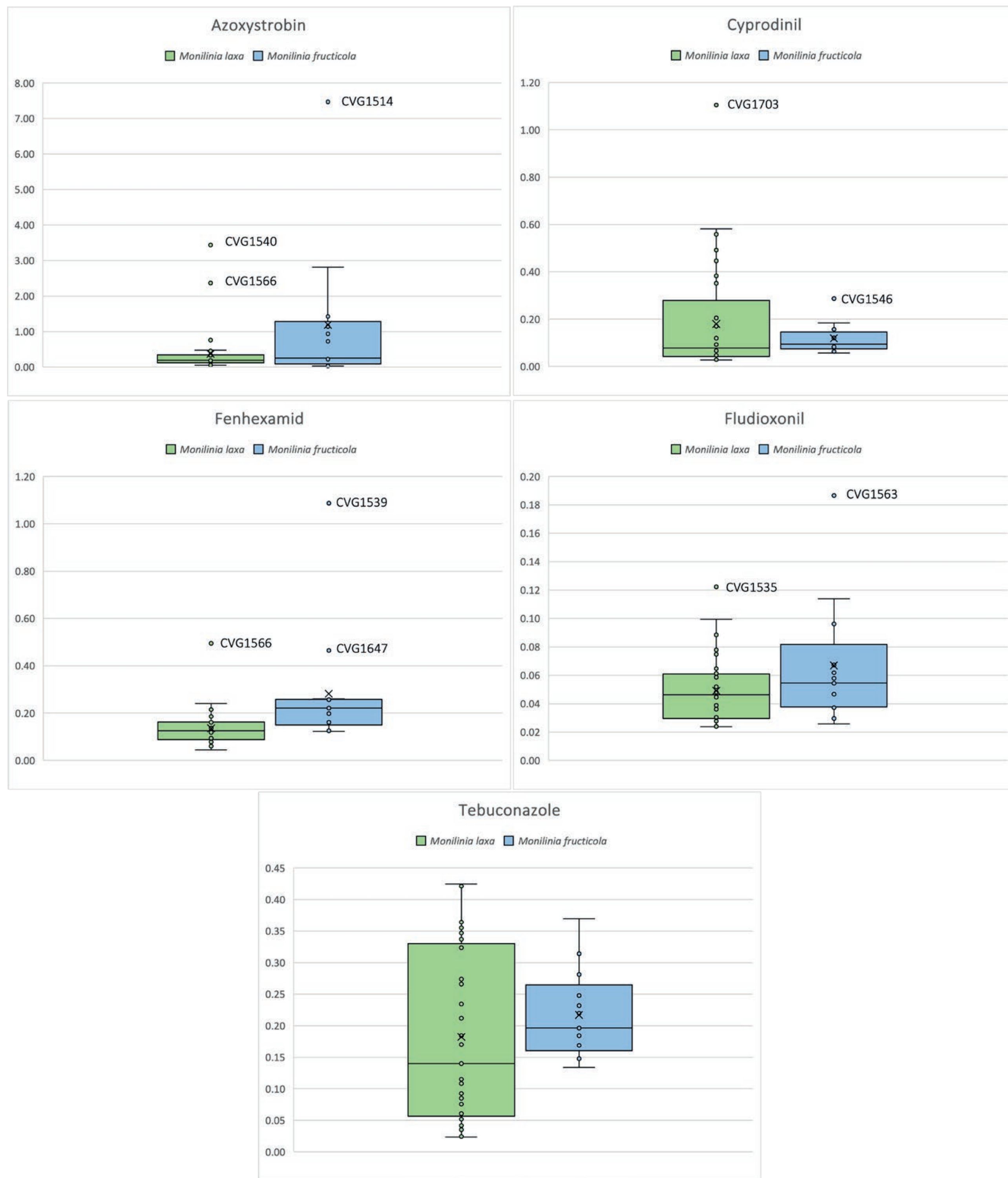

Figure 2. Boxplots of EC_{50s} (μg mL^{-1}) for activities of five fungicides against isolates of *Monilinia laxa* and *M. fructicola*.

mL^{-1} RF of 25.33. The lowest EC_{50} values for cyprodinil for both species were 0.03 μg mL^{-1}, whereas the greatest EC_{50} for *M. fructicola* was 0.29 μg mL^{-1}. Isolates defined as sensitive for cyprodinil had MICs of 0.3 μg mL^{-1}, while some isolates grew at concentrations up to 100 μg mL^{-1}. RF values greater than 10, were recorded for the

M. laxa isolates CVG 1509, CVG 1544, CVG 1703 and CVG 1709.

None of the assessed isolates showed resistance to fenhexamid. The lowest EC_{50} for this fungicide was for the *M. laxa* isolate CVG 1540 (EC_{50} = 0.04), while the *M. fructicola* isolate CVG 1539 was the least sensitive to fenhexamid (EC_{50} = 1.09 μg mL^{-1}. The greatest fenhexamid EC_{50} for *M. laxa* was 0.49 μg mL^{-1}. MIC of 1 μg mL^{-1} was recorded for the sensitive isolates, while other isolates grew at up to 10 μg mL^{-1} of this fungicide. No isolates had RFs greater than 10.

For fludioxonil, the most sensitive *M. laxa* isolate had an EC_{50} of 0.02 μg mL^{-1}, while the greatest EC_{50} was 0.12 μg mL^{-1}. For *M. fructicola*, isolate CVG 1537 was the most sensitive (EC_{50} = 0.03 μg mL^{-1}), while the least sensitive isolate CVG 1563 had an EC_{50} 0.19 μg mL^{-1}. No isolates had RF values greater than 10.

For tebuconazole, EC_{50} values for *M. fructicola* were from 0.13 to 0.37 μg mL^{-1}, and for *M. laxa* were from 0.02 to 0.42 μg mL^{-1}. Sensitive isolates had MIC values of 1 μg mL^{-1}, while some isolate grew at up to 10 μg mL^{-1} of this fungicide. Three isolates of *M. laxa* (CVG 1709, CVG 1713, CVG 1717) had RF values greater than 10.

DISCUSSION

Use of site-specific fungicides is widespread in Europe, and *Monilinia* spp. resistance to different fungicides has been reported in several countries (Malandrakis *et al*., 2013; Egüen *et al*., 2015; Hrustić *et al*., 2018). Site-specific fungicides are commonly used by Italian stone fruit growers, and chemical control is the most effective strategy for control brown rot caused by *Monilinia* spp., which require a maximum of three field fungicide applications during each production season. In Italy, the predominant *Monilinia* species are *M. laxa*, *M. fructigena* and *M. fructicola* (Montuschi *et al*., 2016).

Phylogenetic analyses based on isolate ITS sequences showed two divergent clusters. One cluster included *M. fructicola* and *M. laxa*, and the second contained *M. fructigena* and *M. polystroma*. *Monilia yunnanensis* and *Botrytis cinerea* formed two outgroup clusters. Based on these results, *M. laxa* (37 isolates) was the most common species found in the sampled orchards, while the other 13 isolates were *M. fructicola*. The coexistence of *M. fructicola* and *M. laxa* was previously reported in other countries, including Spain (Villarino *et al*., 2013), Greece (Papavasileiou *et al*., 2015) and the United States of America (Boehm *et al*., 2001). The present study has shown that both *M. laxa* and *M. fructicola* are present, in the Cuneo province. As a larger number of *M. laxa* than *M. fructicola* isolates were collected, this prevalence could be due to low temperatures that have characterized past production seasons, as *M. laxa* grows more rapidly than *M. fructicola* at low temperatures (Papavasileiou *et al*., 2015). These conditions may have promoted development and spread of *M. laxa* over *M. fructicola*. Further investigations are required with more extensive sampling over consecutive years, to confirm this trend and clarify effects of temperature and climate on prevalence and distribution *Monilinia* spp.

The use of site-specific fungicides increases risks of selection of fungicide-resistant pathogens, with gradual reductions in fungicide efficacy and disease control. *Monilinia* spp. have also been classified by the FRAC as pathogens of moderate risk for development of fungicide resistance. For these reasons, sensitivity was assessed of different *Monilinia* spp. isolates to five fungicides that represent chemical classes widely used in Italy for brown rot control.

Nine isolates of *M. laxa* and two of *M. fructicola* gave RF values greater than 10 for different fungicides. The greatest RF was recorded for azoxystrobin in one *M. fructicola* isolate. To define resistant isolates to azoxystrobin, Amiri *et al.* (2010) and Luo and Schnabel (2008) have suggested 3 μg mL^{-1} as a discriminatory concentration for resistance to this fungicide, so MIC of 3 μg mL^{-1} was set in the present study as the discriminatory dose for azoxystrobin resistance. Since it was not possible to compare different discriminatory doses obtained from different protocols, and as a baseline population was not included, classification of susceptible isolates in the present study was based only on MIC values. These results showed that two *M. fructicola* and two *M. laxa* isolates were resistant to QoIs. These results are similar to those of Hrustić *et al.* (2018), who reported presence of moderately resistant isolates of *M. laxa* and *M. fructicola*.

Several mechanisms of resistance to QoI fungicides have been proposed, but in most cases resistance is due to a single point mutation (G143A) in the mitochondrial *cytochrome b* (*Cytb*) gene that leads to an amino acid change in position 143 from glycine to alanine (Hrustić *et al*., 2018). This mutation has been reported only in fungi without specific introns close to this amino acid position (Grasso *et al*., 2006). This intron is present in *M. laxa* isolates after the position 143, as reported by Miessner and Stammler (2010). Similarly, in *M. fructicola* isolates the intron is also present but is located downstream of the codon for glycine at position 143, suggesting that this point mutation may not lead to QoI resistance in *M. fructicola* (Luo *et al*., 2010). Further investigations are required to elucidate the resistance mechanism in *Monilinia* spp. isolates collected in the present study.

For fenhexamid, Malandrakis *et al.* (2013) reported *M. laxa* isolates with EC_{50}s from 0.02 to 1 μg mL^{-1}, while Förster *et al.* (2007), for *M. fructicola*, reported EC_{50}s ranging from 0.09 to 0.21 μg mL^{-1}. Based on EC_{50} measurements, the present study results showed that the assessed *M. fructicola* isolates were less sensitive to fenhexamid than the *M. laxa* isolates. However, calculations of RFs showed that neither the *M. fructicola* nor the *M. laxa* isolates were resistant to fenhexamid. This is probably because fenhexamid had been used only occasionally against brown rot in the sampled orchards.

Two fludioxonil + cyprodinil applications per year are authorized for control of brown rot of stone fruit in Italy. Fludioxonil EC_{50}s for *M. fructicola* have been reported as from 0.05 to 0.21 μg mL^{-1} (Förster *et al.*, 2007), while only Fazekas *et al.* (2014) have reported reduced sensitivity to cyprodinil for *M. laxa*. Results from the present study showed that most of the tested isolates were susceptible to fludioxonil and cyprodinil. Based on RFs greater than 10, only four *M. laxa* isolates had reduced sensitivity to cyprodinil. For fludioxonil, no isolates showed high RF values, suggesting absence of resistance to this fungicide.

Resistance to DMI fungicides has been detected in *M. fructicola* isolates from peach in the United States of America (Chen *et al.*, 2013; Pereira *et al.*, 2020) and Brazil (Lichtemberg *et al.*, 2016). For tebuconazole, a MIC of 1 μg mL^{-1} has been reported and used as the discriminatory dose. Results obtained for tebuconazole showed high EC_{50}s compared with values reported by May-De Mio *et al.* (2011) and by Pereira *et al.* (2020). Based on calculated RFs, only three isolates of *M. laxa* showed RF >10. In the sampled orchards, tebuconazole has been constantly used in past years (pers. comm. from orchard technicians).

Data obtained in the present study showed that *M. laxa* and *M. fructicola* coexist in stone fruit orchards in Cuneo province of Piedmont, with *M. laxa* being the predominant fungus associated with brown rot. Different levels of sensitivity to the tested fungicides were also recorded within the isolate sets of both of these fungi. However, due to the low number of tested isolates, it is not possible to determine if selection of resistant isolates is occurring in the investigated territory. Including a baseline population is also important for establishing a reference point that allows discrimination of sensitivity levels. In the present study, it was not possible to define appropriate resistance baselines due to the absence of orchards in the sampled area where fungi were not exposed to specific fungicide. Therefore, systematic and widespread sampling should be carried out to determine the resistance levels of *Monilinia* spp. populations in this major stone fruit production area. This should include large numbers of isolates, and appropriate reference isolates or baseline populations. Since chemical control in the field remains the most effective strategy for control of *Monilinia* spp., moderate use of these fungicides is recommended to prevent fungicide resistance and maintain their efficacy against these important pathogens.

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Research Papers

Identification and pathogenicity of *Alternaria* species causing leaf blotch and fruit spot of apple in California

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Summary. In late summer 2020, symptoms of leaf blotch and fruit spot were observed in two different commercial apple orchards (cultivars 'Pink Lady' and 'Modi') in San Joaquin County, California, USA. Ninety *Alternaria* isolates were obtained from symptomatic leaves and fruits collected from the orchards. Based on morphological characteristics of the colonies, sporulation patterns, and conidia, the isolates were preliminarily separated into three morphogroups, tentatively identified as *A. alternata*, *A. tenuissima* and *A. arborescens*. Multi-locus phylogenetic analyses, using nucleotide sequences of plasma membrane ATPase, calmodulin, and Alternaria major allergen genes, showed that the isolates initially identified as *A. tenuissima* clustered with strains of *A. alternata*, following the current taxonomical arrangement of the genus. Pathogenicity tests on detached wounded apple leaves and fruits, using representative isolates of the three morphogroups, fulfilled Koch's postulates. This is the first report of *A. alternata* and *A. arborescens* as causal agents of leaf blotch and fruit spot of apple in California.

Keywords. Etiology, foliar, apple diseases, *Malus domestica*.

INTRODUCTION

Apple (*Malus domestica* Borkh.) production in California covered 4,654 ha in 2021, as the sixth largest apple-producing state in the United States of America (USA) (CDFA, 2022). California produces four main apple cultivars: 'Gala', 'Fuji', 'Granny Smith', and 'Cripps Pink' ('Pink Lady®') (California Apple Commission, 2023). Several fungal diseases affect apple, including diseases of leaves and fruit (e.g. apple scab, powdery mildew, rusts), wood (e.g. European canker, Valsa canker), root and replant diseases (e.g. crown and root rots), and postharvest diseases (e.g. blue mold, gray mold and bull's-eye rot) (Sutton *et al*., 2014).

Apple is susceptible to *Alternaria* species, which can cause different diseases, including leaf blotch, fruit spot, fruit rot, core rot, and moldy core

(Harteveld *et al*., 2013; Sutton *et al*., 2014; Gur *et al*., 2017; Elfar *et al*., 2018b). Alternaria leaf blotch is frequently observed in summer, and is characterized by the presence of small (3 to 5 mm diam.), circular gray to brown necrotic lesions on apple leaves and fruit, often with dark brown to purple margins (Elfar *et al*., 2018a). In severe cases, leaf defoliation of up to 50% can occur in susceptible cultivars (Sawamura, 2014). Symptoms on fruit are uncommon, except in highly susceptible cultivars, such as 'Golden Delicious', 'Starking Delicious', 'Indo', 'Gala', and 'Pink Lady'. Fruit spots are often small, corky, and dark, typically associated with fruit lenticels (Sawamura, 2014; Gur *et al*., 2017). Severe disease outbreaks have been reported on 'Pink Lady' apples in northern Israel, where large lesions and fruit rots have been observed. Multiple lesions on fruit, especially those adjacent to cracks around fruit calices, may coalesce to produce large dark rotted areas, with incidences up to 80% of fruit in some orchards (Gur *et al*., 2017). This disease was named Alternaria fruit rot to differentiate it from Alternaria fruit spot (Gur *et al*., 2018). The status of *Alternaria* diseases affecting apples in California is unknown. However, California is familiar with diseases caused by *Alternaria* species in other fruit and nut crops (Teviotdale *et al*., 2001; Pryor and Michailides, 2002; Zhu and Xiao, 2015; Luo *et al*., 2017; Wang *et al*., 2021).

Alternaria mali (syn. *A. alternata* f. sp. *mali*) is the main cited causal agent of Alternaria blotch of apple in the northern hemisphere and Australia (Filajdić and Sutton, 1991; Ozgonen and Karaca, 2006; Soleinami and Esmaizadeh, 2007; Harteveld *et al*., 2013; Sawamura, 2014; Gur *et al*., 2017). In the USA, *A. mali* was identified in the late 1980s in North Carolina, causing apple leaf blotch (Filajdić and Sutton, 1991). However, studies in in Australia, Chile, France, Italy, and Spain, have shown that several small-spored *Alternaria* species were associated with Alternaria leaf blotch and fruit spot, where *A. alternata*, *A. arborescens*, *A. infectoria*, *A. longipes*, and *A. tenuissima* were identified (Rotondo *et al*., 2012; Harteveld *et al*., 2013; Elfar *et al*., 2018a; Toome-Heller *et al*., 2018; Fontaine *et al*., 2021; Cabrefiga *et al*., 2023). Differences in virulence among *Alternaria* isolates were also detected (Harteveld *et al*., 2014b; Elfar *et al*., 2018a).

Morphological identification of the small-spored species of *Alternaria* is challenging, due to the diversity and scarcity of characteristics that allow unambiguous identification (Andrew *et al*., 2009). Use of morphological characteristics in combination with phylogenetic analyses based on multiple gene loci are essential for identification of species within small-spored *Alternaria* (Woudenberg *et al*., 2013; Lawrence *et al*., 2016). Several loci have been used in phylogenetic studies of *Alternaria*, including nuclear ribosomal regions (ITS, LSU, and SSU), and protein-coding genes. Lawrence *et al*. (2013), assessed the phylogenetic utility of ten nuclear protein coding loci, and showed that the five most phylogenetically informative loci for *Alternaria* species were ATPase, followed by calmodulin, Alternaria major allergen Alt a1, glyceraldehyde-3-phosphate dehydrogenase, and actin. The least informative loci were beta-tubulin and translation elongation factor 1-alpha. Therefore, ATPase and calmodulin have been suggested as the most appropriate loci for identification of *Alternaria* species (Lawrence *et al*., 2013, 2016).

During late summer of 2020, an outbreak of leaf blotch and fruit spot was observed in two commercial apple orchards in San Joaquin County, California. Up to 30% of leaves and less than 1% of apples were affected by the disease. The objectives of the present study were: (i) to identify and characterize the causal agents of both of these diseases; and (ii) to test the pathogenicity of the putative pathogens on two apple cultivars ('Pink Lady' and 'Fuji').

MATERIALS AND METHODS

Fungal isolations

Symptomatic apple leaf (n = 40) and fruit (n = 10) samples were collected from two orchards, one of the cultivar 'Pink Lady' and the other of 'Modi', located in San Joaquin County, California. The leaves and fruit were surface disinfected by submerging in a 70% ethanol solution for 1 min. Isolations were then carried out from small pieces (2 to 5 mm length) taken from margins between diseased and healthy tissues, which were plated onto potato dextrose agar (PDA; BD Difco) acidified with 92% lactic acid 0.5 mL L^{-1} (APDA). The isolation plates were incubated for 7 to 10 d at room temperature (20 to 22°C). Fungal colonies were preliminarily identified as *Alternaria* species (Simmons, 2007), using colony morphology (colour and texture) and by conidiophore and conidium characteristics. Mycelium from *Alternaria*-like colonies was transferred to fresh APDA plates, and pure cultures of 90 isolates were then obtained by plating a 50 μL of conidial suspension of each isolate on water agar. After 18 h of incubation at room temperature, a single conidium was selected under a stereomicroscope and transferred to a fresh APDA plate. Isolates were then kept on APDA at 5°C for further analyses.

Morphological characteristics of isolates

Colony morphology was characterized in plastic Petri dishes (90 mm diam.) containing either APDA or potato carrot agar (PCA; HiMedia) (Simmons, 2007). The plates were incubated for 7 d at 20 to 22°C, with 8 h light 16 h darkness regimes. Light was provided by daylight fluorescent tubes placed 40 cm above the culture plates. Conidia and conidiophores from three PCA plates per isolate were each mounted on colourless adhesive tape and placed on top of a drop of Shear's mounting medium (10 g potassium acetate, 200 mL glycerin, 300 mL 95% ethanol, 500 mL distilled water), and were then observed under a light microscope at 400× of magnification. Based on their morphology, the isolates were preliminarily classified into three morphogroups (A, B, and C). Nine representative isolates (group A: UCD9582, UCD9584, UCD9600; group B: UCD9588, UCD9620; group C: UCD9590, UCD9593, UCD9603, UCD9643) were selected for further study of their conidiophore and conidium features. Conidiophore (n = 15 per isolate) length and width, cell numbers, and branching were determined. Conidium (n = 50) shape, length, width, and number of transepta were determined. These data were compared with published descriptions of *Alternaria* species (Simmons, 2007).

DNA extraction, PCR amplification and sequencing

Twenty-six *Alternaria* isolates representative of the three morphological groups were selected for molecular identification (Table 1). These groups were established according to similarities in colony morphology and characteristics of their conidiophores and conidia. Total genomic DNA was extracted from 7- to 10-d-old mycelium of each isolate grown on APDA and incubated at 20 to 22°C. Mycelium of each isolate was carefully separated from the agar medium using a sterile scalpel, and was then macerated (6.0 m sec^{-1} for 40 sec) in a tube containing lysis buffer and 1.0 mm glass beads, using a FastPrep-24 (MP Biomedicals). Genomic DNA was extracted using a DNA extraction kit (NucleoSpin Plant II; Macherey-Nagel GmbH & Co. KG). The Alternaria major allergen Alt a1 gene (Alt a1) was amplified using the primer pair Alt-for/Alt-rev (Hong *et al*., 2005), the plasma membrane ATPase gene (ATPase) using pair ATPDF1/ATPDR1, and the Calmodulin (CAL) gene using pair CALDF1/CALDR1 (Lawrence *et al*., 2013). Polymerase chain reactions (PCR) were carried out in a T100™ thermocycler (Bio-Rad). Each reaction had a volume of 25 mL, containing 12.5 µL of GoTaq® Green MasterMix 2X (Promega), 9.3 µL of nuclease-free water, 0.6 µL of a 10-µM solution of each primer, and 2 µL of template DNA. The amplification protocol included preheating for 2 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 45 s at 57°C for Alt a1, 55°C for ATPase, or 54°C for CAL, and extension at 72°C for 90 s, with a final extension for 5 min at 72°C. PCR-amplified products were visualized by electrophoresis in 1% agarose gels with 100X SYBR® Green I nucleic acid gel stain (Sigma-Aldrich), and purified using Exonuclease I and Shrimp Alkaline Phosphatase (New England BioLab), following the manufacturer's instructions. PCR products were quantified using a Quantus™ fluorometer (Promega), and were submitted to Quintara Biosciences (Hayward, CA, USA) for Sanger sequencing. Both forward and reverse sequences were assembled using Sequencher v5.4.6 (Gene Codes). A BLASTn search analysis of the consensus sequences was carried out against reference sequences in the GenBank database (https:// www.ncbi.nlm.nih.gov).

Phylogenetic analyses

Maximum parsimony (MP) phylogenetic analyses were carried out using MEGA v.11 (Tamura *et al*., 2021). Gaps were treated as missing data. The MP trees were obtained using the tree-bisection-reconnection branch swapping algorithm and 1,000 random sequence additions. Branch stability was estimated using bootstrap with 1,000 replicates. The alignments included sequences of Alt a1, ATPase, and CAL, from the 26 *Alternaria* isolates obtained from apple leaf blotch and fruit spot symptoms in California (Table 1) and sequences from 18 *Alternaria* isolates obtained from GenBank (Table 2). Sequences of *Stemphylium botryosum*, *S. callistephi*, and *S. vesicarium* were included as outgroups (Table 2). The phylogenetic analyses were carried out independently for each gene, and concatenated. Topology of the resulting trees was compared, and a consensus tree was selected. This tree was edited in TreeGraph v2, and visual edits were carried out in InkScape.

Pathogenicity tests

Six representative isolates were selected to test their pathogenicity, two from each of the three morphological groups: group A (isolates UCD9582 and UCD9600), B (UCD9588 and UCD9620), and C (UCD9590 and UCD9593). To stimulate sporulation, isolates were cultivated in 0.05× PDA (Pryor and Michailides, 2002) for 7 d at 20 to 22°C with cycles of 10 h of light and 14 h of darkness. Conidial suspensions were prepared from 10-

Table 1. Sources of isolates of *Alternaria* species from apple obtained in two commercial orchards in Stockton, California, and GenBank accession numbers for sequences of three genes (*Alternaria* major allergen Alt a1, plasma membrane ATPase, and Calmodulin) of the *Alternaria* isolates examined in this study.

Isolate	Group	Species	Symptom [a]	Apple Cultivar	GenBank accession number [b]		
					Alt a1	ATPase	Calmodulin
UCD9582[cd]	A	*Alternaria alternata*	FS	P. Lady	MW685776	MW685792	MW685808
UCD9584[d]	A	*A. alternata*	FS	P. Lady	MW685775	MW685791	MW685807
UCD9598	A	*A. alternata*	LB	P. Lady	MW685777	MW685793	MW685809
UCD9598.2	A	*A. alternata*	LB	P. Lady	MW685778	MW685794	MW685810
UCD9600[cd]	A	*A. alternata*	LB	P. Lady	MW685774	MW685790	MW685806
UCD10530	A	*A. alternata*	LB	Modi	OQ803488	OQ803499	OQ803510
UCD10533	A	*A. alternata*	LB	Modi	OQ803489	OQ803500	OQ803511
UCD10536	A	*A. alternata*	LB	Modi	OQ803490	OQ803501	OQ803512
UCD10539	A	*A. alternata*	LB	Modi	OQ803491	OQ803502	OQ803513
UCD10529	B	*A. alternata*	LB	Modi	OQ803492	OQ803503	OQ803514
UCD9588[cd]	B	*A. alternata*	FS	P. Lady	MW685789	MW685805	MW685821
UCD9620[cd]	B	*A. alternata*	LB	P. Lady	MW685788	MW685804	MW685820
UCD9590[cd]	C	*A. arborescens*	FS	P. Lady	MW685782	MW685798	MW685814
UCD9591	C	*A. arborescens*	FS	P. Lady	MW685783	MW685799	MW685815
UCD9593[cd]	C	*A. arborescens*	LB	P. Lady	MW685781	MW685797	MW685813
UCD9603[d]	C	*A. arborescens*	LB	P. Lady	MW685780	MW685796	MW685812
UCD9643[d]	C	*A. arborescens*	LB	P. Lady	MW685779	MW685795	MW685811
UCD9643.2	C	*A. arborescens*	LB	P. Lady	MW685784	MW685800	MW685816
UCD9644	C	*A. arborescens*	LB	P. Lady	MW685785	MW685801	MW685817
UCD9645	C	*A. arborescens*	LB	P. Lady	MW685786	MW685802	MW685818
UCD10531	C	*A. arborescens*	LB	Modi	OQ803493	OQ803504	OQ803515
UCD10532	C	*A. arborescens*	LB	Modi	OQ803494	OQ803505	OQ803516
UCD10534	C	*A. arborescens*	LB	Modi	OQ803495	OQ803506	OQ803517
UCD10535	C	*A. arborescens*	LB	Modi	OQ803496	OQ803507	OQ803518
UCD10537	C	*A. arborescens*	LB	Modi	OQ803497	OQ803508	OQ803519
UCD10538	C	*A. arborescens*	LB	Modi	OQ803498	OQ803509	OQ803520

[a] FS = fruit spot, LB = leaf blotch.
[b] Genes: Alt a1 = Alternaria major allergen Alt a1, ATPase = plasma membrane ATPase.
[c] Isolates used for pathogenicity tests on apple fruit and leaves.
[d] Isolates used for morphological characterization.

to 14-d-old cultures. Plates were flooded with approx. 20 mL of 0.05% Tween 80 and the medium surface in each plate was scraped with a sterile scalpel. The resulting conidial suspension was filtered through four layers of gauze and the concentration was adjusted to 1×10^5 conidia mL^{-1}, using a haemocytometer for conidia counting.

Apple leaves. Detached fully expanded mature leaves from 'Pink Lady' and 'Fuji' apple (n = 10 from each cultivar) were surface disinfected in 1% NaOCl for 1 min, followed by sterile distilled water for 1 min, and were then air dried inside a laminar flow hood. Nine punctures were made on each leaf, three punctures were made on the apical, basal, and middle regions, using a sterile hypodermic needle (31G). Leaves were inoculated by placing 15 µL of conidial suspension on top of each wound site. The leaves were then incubated at 20°C in humid chambers for 7 d until symptoms development. Evaluations were carried out by measuring the lesion diameters using a digital caliper. An equal number of wounded leaves treated with sterile water were included as negative controls. Re-isolations from resulting necrotic lesions were made onto APDA, and obtained colonies were identified based on the conidia morphology. The experiment was conducted twice.

Apple fruit. Mature fruit (mean total soluble solids 14.2%) of 'Pink Lady' and 'Fuji' apple (n = 10 of each cultivar) were surface disinfected in 70% ethanol for 5 min and air dried inside a laminar flow hood. Each fruit was then inoculated with 20 µL of a conidial sus-

Table 2. Accession numbers for reference sequences of *Alternaria* isolates in GenBank used for phylogenetic analyses in this study.

Species	Isolate[b]	GenBank accession number[a]		
		Alt a1	ATPase	Calmodulin
Alternaria alstroemeriae	CBS 118809	MH084526	MH101803	MH175185
A. alternata	EGS 34-016	KP275691	JQ671874	JQ646208
A. alternata (=*A. angustiovoidea*)	EGS 36-172	JQ646398	JQ671869	JQ646203
A. alternata (=*A. destruens*)	EGS 46-069	JQ646402	JQ671873	JQ646207
A. alternata (=*A. dumosa*)	EGS 45-007	AY563305	JQ671877	JQ646211
A. alternata (=*A. herbiphorbicola*)	EGS 40-140	JQ646410	JQ671888	JQ646222
A. alternata (=*A. limoniasperae*)	EGS 45-100	JQ646370	JQ671879	JQ646213
A. alternata (=*A. tenuissima*)	EGS 34.015	KP275690	JQ811989	JQ646209
A. arborescens	EGS 39-128	AY563303	JQ671880	JQ646214
A. arborescens	3.J24	KJ921023	KJ908244	KJ920979
A. arborescens (=*A. cerealis*)	EGS 43-072	JQ646405	JQ671883	JQ646217
A. argyroxiphii	EGS 35-122	JQ646434	JQ671926	JQ646260
A. betae-kenyensis	CBS 118810	KP123966	MH101805	MH175189
A. eichhorniae	CBS 489.92	KP123973	MH101806	MH175190
A. gossypina	CBS 104.32	JQ646395	JQ671868	JQ646202
A. grossulariae	CBS 100.23	JQ646394	JQ671867	JQ646201
A. jacinthicola	CBS 133751	KP123984	MH101793	MH175187
A. tomato	CBS 114.35	JQ646389	JQ671861	JQ646195
Stemphylium botryosum	ATCC 42170	AY563274	JQ671767	JQ646101
S. callistephi	EEB 1055	AY563276	JQ671769	JQ646103
S. vesicarium	ATCC 18521	AY563275	JQ671768	JQ646102

[a] Genes: Alt a1 = Alternaria major allergen Alt a1, ATPase = plasma membrane ATPase.
[b] ATCC = American Type Culture Collection, Manassas, VA; CBS = Centraalbureau voor Schimmelcultures, Royal Netherlands Academy of Arts and Sciences, Utrecht, the Netherlands; EEB, E. E. Butler, Department of Plant Pathology, University of California, Davis, CA; EGS = E. G. Simmons, Mycological Services, Crawfordsville, IN.; 3.J = Pryor and Michailides 2002.

pension that was deposited on top of four punctures made with a sterile hypodermic needle (31G). Fruits were then incubated at 20°C inside humid chambers for 14 d until symptom development. Resulting necrotic lesions were measured using a digital caliper. An equal number of wounded fruits treated with sterile distilled water were included as negative controls. Prior to determining the necrotic lesions, each fruit was cut vertically through each wound with a sterile knife, and the necrotic lesion length inside the fruit from the wound was measured. Re-isolations from the necrotic lesions were carried out on APDA to determine fulfilment of Koch's postulates, and obtained colonies were identified based on the conidia morphology. This experiment was conducted twice.

Experimental designs and statistical analyses

Pathogenicity test experiments were carried out according to a 2 × 6 (apple cultivar × isolate) factorial design, with ten replicates, each of one fruit or one leaf as the experimental unit. Lesion diameters and lengths were subjected to analysis of variance (ANOVA) using generalized linear models with the corresponding R packages in InfoStat v 2008. Means were separated using Fisher's least significant difference test ($P < 0.05$).

RESULTS

Symptoms and fungal isolations

During the disease outbreak, symptoms were mainly observed on apple leaves, and were characterized by the presence of one or more circular brown necrotic lesions (each 2 to 15 mm diam.) per leaf, with each lesion enlarged in zonate circular or crescent-shaped rings, and often with a dark brown to purple margin. With time, the affected leaves turned yellow and fell prematurely. On fruits, rounded, dark-coloured, dry, corky lesions (each 2 to 30 mm diam.) were observed (Figure 1).

Colonies of isolated fungi on APDA were gray-green to dark olive green with whitish margins. All *Alternaria* isolates produced single conidiophores and catenulate brown to golden-brown conidia.

Figure 1. Alternaria leaf blotch and fruit spot on 'Pink Lady' apple. **A** and **B**, naturally infected leaves. **C**, A naturally infected fruit.

Morphological characteristics of isolates

Preliminary categorization of the isolates based on colony morphology and sporulation patterns (Simmons, 2007), placed 36% of the isolates in group A, 18% in group B, and 47% in group C.

Group A isolates were tentatively identified as *A. alternata*, with these isolates producing cottony, gray to green colonies with white margins on PDA. Conidia chains were observed along with numerous secondary chains branching on short conidiophores. Conidia were ovoid to ellipsoid, and had average dimensions of 26.8 ± 4.63 × 9.8 ± 1.3 μm (Figure 2, A, D and G).

Group B isolates were morphologically identified as *A. tenuissima*, with these isolates producing cottony, gray olive brown colonies with slight concentric growth rings and white margins on PDA. Conidia chains had between six and 14 conidia, rarely with a lateral branch. Conidia were ovoid to obclavate, each with a narrow tapered upper half, and the conidia had average dimensions of 26.7 ± 5.1 × 9.5 ± 1.2 μm (Figure 2, B, E and H).

Group C isolates were identified as *A. arborescens*. These isolates produced cottony, olive-brown colonies with concentric growth rings, often with wavy margins on PDA. The conidiophores were long and had extended secondary conidiophores. Conidia chains had two to seven conidia, and conidium development was concentrated near the apices of secondary, tertiary, and quaternary conidiophores. Conidia were ovoid to ellipsoid, and had average dimensions of 26.2 ± 6.0 × 9.0 ± 1.3 μm (Figure 2, C, F and I).

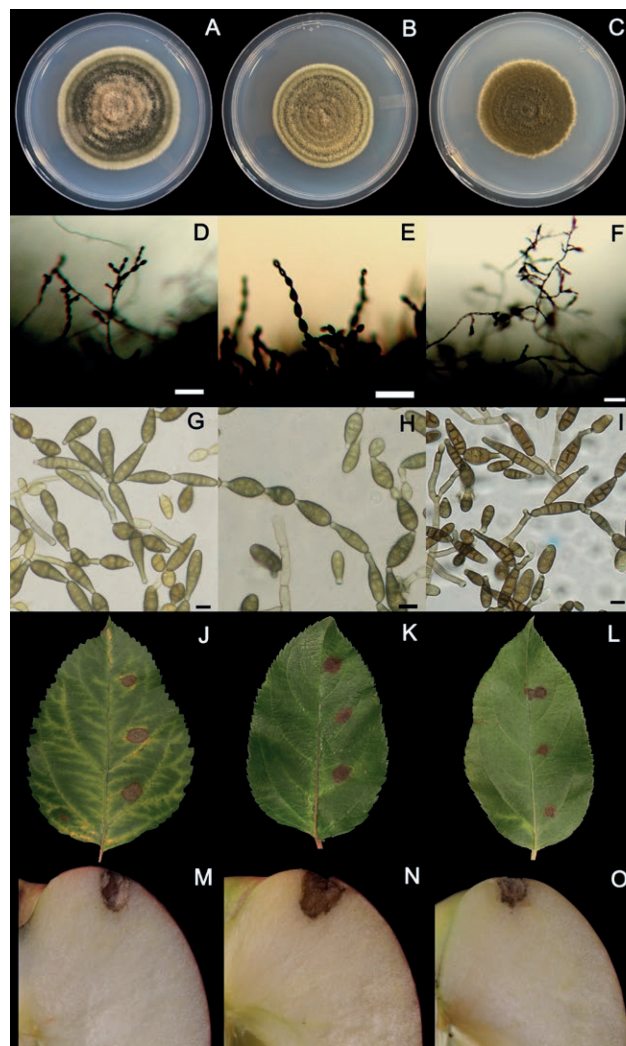

Figure 2. Morphological features of *Alternaria* species isolated from apple. **A** to **C**, Colony morphologies on PDA after 7 d incubation at 22°C under 8 h light/16 h dark regime; **D** to **F**, Sporulation patterns on PCA after 7 d incubation at 22°C under 8 h light/16 h dark regime; **G** to **I**, conidia; **J to L**, symptoms on inoculated 'Pink Lady' leaves; **M** to **O**, symptoms on inoculated 'Pink Lady' fruit cut vertically; **A, D, G, J,** and **M,** isolate of group A (UCD9582); **B, E, H, K, N,** isolate of group B (UCD9588); **C, F, I, L, O,** isolate of group C (UCD9593). White scale bars = 50 μm, black bars = 10 μm. Based on morphology and phylogeny, isolates in group A and B were identified as *A. alternata*, and isolates in group C were identified as *A. tenuissima*.

Phylogenetic analyses

The consensus sequence length of Alt a1 was 472 bp, of ATPase, was 1,194 to 1,197 bp, and CAL was 718 to 723 bp. The maximum parsimony analyses of Alt a1 (346 character dataset after alignment), ATPase (1,220 character dataset after alignment), and CAL (770 character dataset after alignment) combined produced a consensus tree (Figure 3) from the 25 most parsimonious trees (tree length = 731, consistency index = 0.804, retention index = 0.880, rescaled consistency index = 0.708). All the 26 *Alternaria* isolates obtained clustered with isolates that belong to the section *Alternaria*. The phylogenetic tree showed that there was clear separation of group C isolates from those of groups A and B. Isolates from group C clustered (96% bootstrap support) with the ex-type strain of *A. arborescens* (EGS 39128) and other *A. arborescens* reference isolates (Figure 3). Isolates from groups A and B, despite their morphological differences, were clustered with the *A. alternata* ex-type (EGS 40140) and other *A. alternata* reference isolates (Figure 3). Of the 26 *Alternaria* isolates obtained from apple fruits and leaves in California, 12 were identified as *A. alternata* (46%), and 14 corresponded to *A. arborescens* (54%).

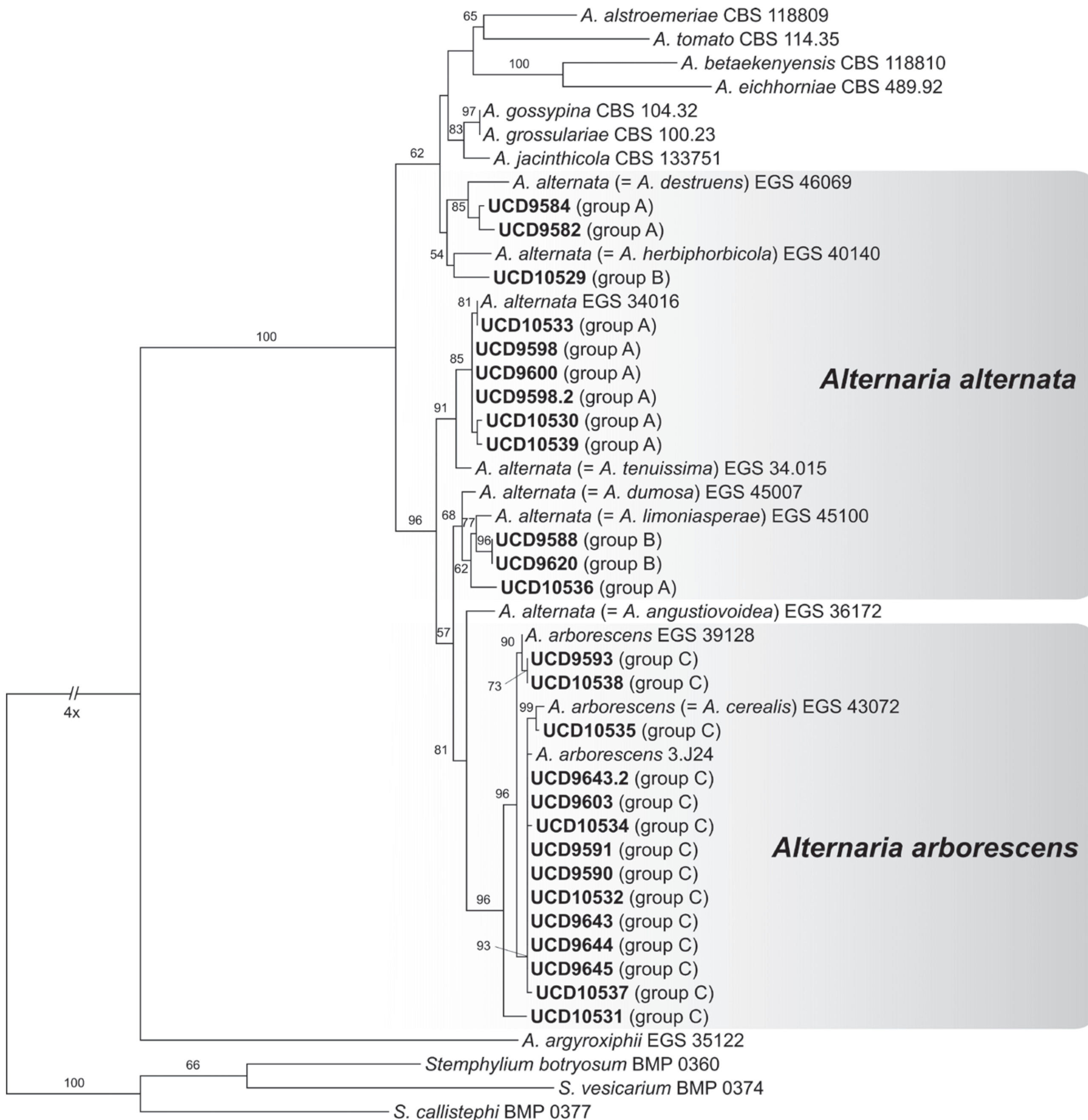

Figure 3. Phylogenetic tree obtained from maximum-parsimony analysis of the Alternaria major allergen Alt a1, plasma membrane ATPase, and calmodulin gene sequences of *Alternaria* species from Californian apple and sequences of ex-types in GenBank. The consensus tree shown is inferred from the 25 most parsimonious trees and bootstrap values obtained. The tree was rooted with *Stemphylium botryosum*, *S. callistephi*, and *S. vesicarium*. Tree length = 731, consistency index = 0.804, retention index = 0.880, and rescaled consistency index = 0.708. UCD and numbers (in bold) are *Alternaria* isolates from apple in California; other codes are isolates from GenBank.

Pathogenicity tests

All the tested isolates of *A. alternata* and *A. arborescens* were pathogenic on detached apple leaves, which developed brown necrotic lesions of 3.8 to 8.8 mm diameters after 7 d at 20 to 22°C, with the lesions developing concentric rings as they grew (Figure 2, J to L). Differences in disease severity caused by the *Alternaria* isolates were statistically significant ($P < 0.001$) for lesion diameter, but cultivar did not affect this parameter ($P =$

Table 3. Pathogenicity of *Alternaria* isolates studied on leaves and fruits of apple cultivars 'Pink Lady' and 'Fuji', assessed from dimensions of necrotic lesions and dry rots developed after controlled inoculations.

Group	Species	Isolate	Leaves, mean necrotic lesion dimensions (mm) [ab]				Fruit, mean dry rot lesion dimensions (mm) [ac]					
			'Pink Lady'	'Fuji'	Mean		'Pink Lady'		'Fuji'		Mean	
A	*A. alternata*	UCD9600	6.6	8.8	7.7	a	3.9		9.1		6.5	a
		UCD9582	7.5	6.7	7.1	a	4.2		7.3		5.7	ab
B	*A. alternata*	UCD9620	6.5	6.5	6.5	a	5.2		5.3		5.2	ab
		UCD9588	7.6	5.4	6.5	a	4.7		4.7		4.7	ab
C	*A. arborescens*	UCD9593	3.8	4.3	4.0	b	4.1		4.2		4.1	b
		UCD9590	5.4	6.5	5.9	a	3.2		4.6		3.9	b
	Mean		6.2	6.4			4.2	B	5.8	A		

Analysis of variance

	df	F	P	SED	df	F	P	SED
Isolate (I)	5	5.13	<0.001	0.926	5	2.37	0.044	0.849
Cultivar (C)	1	0.02	0.889	0.552	1	9.54	0.003	0.509
I × C interaction	5	1.32	0.259	1.308	5	1.77	0.125	1.089

[a] Non-inoculated controls remained symptomless, and these data were excluded from statistical analyses. Means (each of ten replicates) followed by the same letter in each column are not different (Fisher LSD test, $P = 0.05$). SED = standard error of the difference (standard error of the mean × $\sqrt{2}$).

[b] Leaves were inoculated with conidial suspensions (10^5 conidia mL^{-1}), and lesion diameters were determined after 7 days at 20°C in humid chambers.

[c] Fruit were each inoculated with 20 μL of conidial suspension (10^5 conidia mL^{-1}), then incubated in humid chambers at 20°C for 14 d.

0.889). The isolate × cultivar interaction was also non-significant ($P = 0.259$). The different *Alternaria* isolates had similar virulence (mean lesion diameter = 6.7 mm), except for *A. arborescens* isolate UCD9593, which was the least virulent isolate, causing the smallest lesions (mean = 4.0 mm) (Table 3).

Regardless of the *Alternaria* species, all the isolates caused dry rot on the epidermis and pulp of mature apple fruits after conidia inoculations. Symptoms consisted of dark-coloured, dry, corky lesions of lengths 3.9 to 6.5 mm (Figure 2, M to O). Significant differences ($P < 0.05$) in virulence were observed among the *Alternaria* isolates. The most virulent isolate was *A. alternata* isolate UCD9600 (mean lesion length = 6.5 mm), whereas *A. arborescens* isolate UCD9590 was the least virulent (mean = 3.9 mm). Apple cultivar had significant effects ($P < 0.01$) on lengths of the dry rot lesions, with 'Fuji' being more susceptible (mean = 5.8 mm) than 'Pink Lady' (mean = 4.2 mm). The interaction isolate × cultivar was non-significant ($P = 0.125$) (Table 3).

Re-isolations from the margins of the necrotic lesions and dry rots were accomplished from all of the inoculated leaves and fruits. Identifications of the re-isolated fungi was confirmed morphologically as those of the inoculated fungi. Non-inoculated leaves and fruits remained symptomless. These results fulfilled Koch's postulates for all the inoculated isolates.

DISCUSSION

This study is the first to demonstrate that Alternaria leaf blotch and fruit spot are two diseases occurring in California apple orchards, and that both diseases are caused by two small-spored *Alternaria* species, *A. alternata* and *A. arborescens*. The fungi were identified by their morphological features and nucleotide sequences of three DNA barcodes (Lawrence *et al.*, 2013; Pryor and Michailides, 2002; Simmons, 2007; Woudenberg *et al.*, 2015).

There is consensus that identification of small-spored *Alternaria* species is difficult due the few morphological or molecular characteristics that allow species discrimination. Previous studies have demonstrated that host-specificity and geographic associations are not useful characters for *Alternaria* classification, and that morphological classifications are poor predictors of phylogenetic relationships among small-spored *Alternaria* taxa, especially due to high levels of morphological plasticity between and within *Alternaria* sections (Serdani *et al.*, 2002; Andrew *et al.*, 2009; Lawrence *et al.*, 2016).

Based on morphological characteristics of the colonies, sporulation patterns, and conidia, the isolates obtained in the present study from symptomatic apple leaves and fruit were grouped into three morphotypes (A, B and C). These groups were preliminarily identified, respectively, as *A. alternata*, *A. tenuissima* and *A. arborescens*. However, the multi-locus phylogenetic analyses using Alt a1, ATPase, and CAL sequences revealed that the isolates of group C formed a clear separate cluster with reference strains of *A. arborescens*. This is unlike the *A. alternata* isolates (group A) and *A. tenuissima* isolates (group B), which grouped together, despite their morphological difference in conidia chains that allow distinction between these two species (Simmons, 2007). Isolates of group B had long unbranched conidia chains, which is a key morphological characteristic for the identification of *A. tenuissima*. Similarly, in previous studies (Andrew *et al*., 2009; Wang *et al*., 2021), isolates morphologically classified as *A. alternata* and *A. tenuissima* were genetically indistinguishable using multiple molecular markers (*endoPG, OPA1-3*, and *OPA10-2*, or *ATPase*, *CAL*, and *rpb2* genes), and many other isolates were assigned as intermediates between the two groups. Based on genome and transcriptome comparisons and molecular phylogenies, Woudenberg *et al*. (2015) synonymized 35 morphospecies, which cannot be distinguished based on their multi-gene phylogenies, under *A. alternata*, including *A. tenuissima*. *Alternaria mali* was also synonymized with *A. alternata*, but *A. alternata* f. sp. *mali* is currently recognized for isolates which produce the host-specific AM-toxin. Consequently, in the present study, the isolates from group B (preliminarily as *A. tenuissima*) were then identified as *A. alternata*, along with the isolates from group A. However, there is still room for further investigation to determine presence of the AM-toxin gene and to verify their ability to produce AM-toxin.

In the last ten years, only small-spore *Alternaria* species have been described causing Alternaria leaf blotch and fruit spot of apple. *Alternaria alternata* and *A. arborescens* have been the most prevalent in different growing regions, including Australia (Harteveld *et al*., 2013), Chile (Elfar *et al*., 2018a), France (Fontaine *et al*., 2021), Italy (Rotondo *et al*., 2012), New Zealand (Toome-Heller *et al*., 2018), and Spain (Cabrefiga *et al*., 2023). These studies indicate that *A. alternata* and *A. arborescens* are the main causal agents of Alternaria leaf blotch and fruit spot in these regions, and that both species coexist in the same orchards (Fontaine *et al*., 2021). Additionally, these fungi are known to be well distributed on flowers and fruits from early season to harvest, serving as potential inoculum sources (Niem *et al*., 2007; Elfar *et al*., 2019). In the San Joaquin Valley of California, *A. alternata* and *A. arborescens* have been reported as the most prevalent species associated with Alternaria diseases in other fruit and nut crops, including Alternaria leaf spot of almond (Teviotdale *et al*., 2001), fruit rot of blueberry (Zhu and Xiao, 2015), fruit rot of mandarin (Wang *et al*., 2021), heart rot of pomegranate (Luo *et al*., 2017), and Alternaria late blight of pistachio (Pryor and Michailides, 2002). Therefore, the present study corroborates that both of these species are well adapted to the environmental conditions of the Central Valley, and that susceptible crops constitute inoculum sources of *Alternaria* species. Additionally, in the USA, specifically on the East Coast, there are reports of Alternaria leaf spots in field crops (Filajdić and Sutton, 1991) and postharvest fruit spots caused by *Alternaria* species (Jurick II *et al*., 2014; Kou *et al*., 2014).

Pathogenicity tests on detached leaves and fruits are common and efficient practices to fulfill Koch's postulates for *Alternaria* species on apples (Harteveld *et al*., 2014b; Gur *et al*., 2017; Elfar *et al*., 2018a; Toome-Heller *et al*., 2018; Fontaine *et al*., 2021). Rotondo *et al*. (2012) concluded that bioassays on detached leaf tissues were reproducible with unambiguous symptoms. Furthermore, greater proportions of lesions developed on wounded than on nonwounded leaves (Rotondo *et al*., 2012). Similar results have been observed on different crops. Examples include pistachios, where unwounded inoculated leaves did not develop substantial lesions (Pryor and Michailides, 2002), and on *Amaranthus hybridus*, where nonwounded inoculated leaves remained asymptomatic (Blodgett and Swart, 2002). However, *Alternaria* species are capable of infecting and colonizing healthy leaves, and these infections generally remain latent until leaf defenses are compromised, making them more susceptible due to injury, stress, or senescence (Blodgett and Swart, 2002; Pryor and Michailides, 2002; Rotondo *et al*., 2012).

Harteveld *et al*. (2014b) determined that regardless of the *Alternaria* species and the symptom they were originally obtained from (leaf blotch or fruit spot), all isolates were pathogenic on detached nonwounded leaves. However, not all the tested isolates caused fruit spots on attached nonwounded fruits, regardless of the symptom they were recovered from. None of the *A. arborescens* isolates they studied were pathogenic on fruits. Therefore, differential tissue specificity probably occurs across isolates. Similarly, Elfar *et al*. (2018b) found that *Alternaria* isolates obtained from leaf blotch symptoms were incapable to produce symptoms on fruits. In the present study, all the isolates of *A. alternata* and *A. arborescens* were pathogenic on leaves and fruits,

regardless of the isolate or the symptom it was obtained from. Statistically significant differences ($P < 0.001$) were detected between isolates for lesion diameters, indicating differences in virulence. Similar results have been described in previous studies, which suggest that pathogenicity is isolate-dependent rather than species-dependent (Rotondo *et al*., 2012; Harteveld *et al*., 2014b; Fontaine *et al*., 2021).

This study is the first to identify *Alternaria* species causing leaf blotch and fruit spot of apple in California, although a larger scale survey is required to establish the importance and extent of these pathogens. Based on the our results, prevalence of leaf blotch was up to 30%, which is greater than that in compared to Chile, where the observed prevalence was from 0.1 to 4.0% (Elfar *et al*., 2018a). However, the prevalence of fruit spot in the present study was less than 1%, which is similar to that reported in Australia (< 2%) (Harteveld *et al*., 2014a). In Israel, high prevalence levels have been recorded after severe outbreaks of Alternaria leaf blotch and fruit spot in 'Pink Lady' orchards, with up to 80 % of the fruit affected (Gur *et al*., 2017). Therefore, Californian isolates may be less virulent than the Israeli isolates, or the environmental conditions in California are less conducive for the development of these diseases. Epidemiological studies have shown that the diseases develop when temperatures range between 12 and 28°C, and the severity of Alternaria leaf spot increases with increasing duration of moisture (Filajdić and Sutton, 1992). These conditions coincide with the conidia release, which commences when median temperatures exceed 12.5 °C in association with precipitation events (Cabrefiga *et al*., 2023). At the optimum temperature (23.5°C) only 5.1 h of wetness were required for light infections, and 12.7 h for severe infections (Filajdić and Sutton, 1992). In the San Joaquin Valley, springs (April and May) are characterized by high rainfall and moderate temperatures (15 to 19°C), and these are followed by dry summers (June and August) with temperatures between 25 and 33°C (Mila *et al*., 2005). Therefore, the risks of severe Alternaria leaf blotch and fruit spot outbreaks are likely to be low, due to the absence of rainy days during summer. However, the presence of overhead sprinklers used by growers during the summer could be a predisposing factor for the development of Alternaria leaf blotch and fruit spot of apple.

The present study is the first to report *A. alternata* and *A. arborescens* associated with apple leaf blotch and fruit spot in California. Currently, these are considered as minor apple diseases in this state. However, the present results do not exclude the possibility that other *Alternaria* species may be associated with leaf spot and fruit spot of apple in California. Furthermore, these results serve as a starting point for understanding etiology of these diseases, and establishing disease management strategies in case outbreaks occur when predisposing conditions are present.

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Short Notes

Pathogenicity of *Botryosphaeriaceae* and *Phytophthora* species associated with *Paulownia* dieback, canker and root rot in Italy

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Summary. In recent years, an unusual decline and mortality has been observed in *Paulownia* plantations throughout the Marche region (Central Italy). Given the economic importance of this emerging forest crop, a study was conducted to determine which pathogens are directly involved in this syndrome. Field surveys performed in two plantations revealed the widespread occurrence of severe disease symptoms such as leaf chlorosis, crown thinning, shoot and branch dieback, sunken cankers, epicormic shoots and root rot. Disease incidence was also assessed by aerial remote sensing (RS) technologies using drones. Symptomatic samples collected from both stem and root tissues yielded fungal and fungal-like colonies representing two distinct families: *Botryosphaeriaceae* and *Peronosporaceae*. Morphological and DNA sequence data revealed five distinct species, identified as *Macrophomina phaseolina* and *Botryosphaeria dothidea* (*Botryosphaeriaceae*), *Phytophthora pseudocryptogea, P. citrophthora* and *P. erythroseptica* (*Peronosporaceae*). Given that all species are reported here for the first time on Paulownia, Koch's postulates were satisfied inoculating the three *Phytophthora* species and two *Botryosphaeriaceae* at the collar of the stem of potted 1-year-old rooted cuttings in June 2023. Thirty days after inoculation, all plants showed the same symptoms as those observed in the field.

Keywords: *Phytophthora pseudocryptogea*, *P. citrophthora*, *P. erythroseptica*, *Macrophomina phaaseolina*, *Botryosphaeria dothidea*, emerging diseases.

INTRODUCTION

Paulownia spp., autochthonous and deciduous tree species from China, were, in few decades, rapidly introduced in different environments around the world, including Australia, USA, Asia, Europe, and Central Africa (Muthuri *et al*., 2004; Jakubowski, 2022). Paulownia popularity is ascribed to: i) the capability to grow in poor soil and marginal lands, ii) quick growing through a cultivation system called short rotation coppice, which allows

more harvests during their production cycle in a shorter time than traditional forest species (Hauk *et al*., 2014; Vanbeveren *et al*., 2017), iii) high carbon sequestration (Basu *et al*., 2016), and iv) high value and flexibility of wood, and eco-sustainable and alternative energy sources (Testa *et al*., 2022). For these beneficial characteristics, paulownia was considered in the first years as a promising crop, adapted for any soil types, and any environmental conditions.

Concerns about the ecological impact of Paulownia introduction arose recently, when in some countries, *Paulownia tomentosa* has been declared dangerous, and it has been recognized as an invasive species in Austria (Botond and Botta-Dukát, 2004; Essl, 2007). The invasiveness of *P. tomentosa*, and to a lesser rate *P. elongata*, is due to the ability to propagate vegetatively by suckering and resprouting after cutting, as well as to an impressive reproductive potential (Jakubowski, 2022). Sterile clones have been selected to prevent these plants from becoming invasive in new areas where extensive commercial plantings for wood production are established.

Furthermore, several researches focused on paulownia cultivation demonstrating that biomass production is particularly high in optimal conditions, but it resulted directly affected by stationary conditions (i.e. drought, salinity, pH value, temperature, nutrient content) (Ivanova *et al*., 2016; Sage and Sultmanis, 2016; Wang *et al*., 2019; Wozniak *et al*., 2022).

A further impact is due to several pathogens, which can take advantage of stress conditions or can directly come from the nursery, where propagation for many clones is via *in vitro*, rarely by seeds, and more frequently by root cuttings (Stuepp *et al*., 2015; Pozoga *et al*., 2019; Temirov *et al*., 2021).

The most well-known phytosanitary problems on *Paulownia* spp. are witches' broom determined by phytoplasma (Gao *et al*., 2008), Phytophthora root and collar rot (Aloi *et al*., 2021), wood decay caused by *Trametes hirsuta* (Milenkovic *et al*., 2018) and root-knot nematodes (Skwiercz *et al*., 2019). Other diseases affecting *Paulownia* spp. are blight caused by *Alternaria*, Paulownia scab caused by *Elsinoe ampelina* and *Sphaceloma paulowniae*, leaf spot caused by *Phyllosticta* sp., leaf brown spot caused by *Cercospora* sp., and canker caused by *Valsa paulowniae* (Ray *et al*., 2005; Pleysier *et al*., 2006; Pasiecznick, 2019; Liu *et al*., 2022).

Given the growing expansion of decline and mortality events in several paulownia plantations in central Italy and the lack of information available about the aetiology, the goal of this present study was to isolate and identify the main pathogens associated with the disease, as well as to test their pathogenicity.

MATERIAL AND METHODS

Study site, field surveys and decline assessment

From spring 2022 to spring 2023, the phytosanitary status of two 6-year-old plantations of *Paulownia elongata* × *P. fortune* hybrid was monitored for the occurrence of disease symptoms. The two plantations of about 3 and 1 ha, respectively, are located in the province of Pesaro Urbino (central-eastern Italy) and are characterized by tree spacing of 4 × 6 m and clay-loam soil. The propagative material was composed of 1-year old cuttings with roots, provided from Germany, and planted in April 2016. The first technical cut was carried out in 2017, one year after planting. No chemical control was applied on the canopy, nor to disinfect the technical cut, nor for the soil management, for which mechanical processing was carried out three times per year. The plantations were not equipped with an autonomous irrigation system, but water was provided when needed according to the trend of climatic conditions.

In 2022, in each plantation, 10 linear transects, consisting of 25 trees/each, were established *at random* and for each tree, dendrometric data (tree diameter and height) and severity of disease symptoms on the canopy including leaf chlorosis, crown thinning, shoot and branch dieback, sunken cankers, epicormic shoots and root rot were assessed according to an empirical scale with four disease severity levels: 0 = healthy plant, 1 ≤ 30%, 2 = 30–50%, 3 ≥ 50%, 4 = dead plant.

In July 2023, the phytosanitary status of the two plantations was monitored and assessed by aerial remote sensing (RS) using drones (Unmanned Aerial Vehicles, UAV), equipped with digital, multispectral, fluorescence sensors that offer finer resolution of plant diseases and assist in plant disease detection at an earlier stage.

The flight was made using a DJI Mavic 3 Multispectral drone. A carefully planned flight path covered the entire study area with an additional 10-metre buffer to ensure complete coverage of all target plants for evaluation. The Real-time Kinematic (RTK) service was utilized, enabling precise positioning and navigation data without the need for Ground Control Points (GCPs). The UAV maintained a constant speed of 3.5 m/s, capturing an image every 3 seconds. The sensors automatically and simultaneously took 5 images: one for RGB representation and the others to record reflectance values in the GREEN, RED, near-infrared (NIR), and RedEdge regions. The RGB images have dimensions of 5280 × 3956 pixels with a bit depth of 24 bits and were acquired with an exposure time of 1/1000 sec. Conversely, the multispectral images are 2592 × 1944 pixels, 16-bit, with an exposure time of 1/640 sec. All

images were acquired at 96 dpi. The focal length for the RGB images was 12 mm, while for the individual band acquisitions, it was 4 mm. The georeferenced orthomosaic processing for each study area was conducted using Agisoft Metashape software. The first study area, covering 0.122 km^2, has a total error of 2.33 cm, with a ground resolution of 49.2 cm/pixel. The orthomosaic of the second study area, covering 0.081 km^2, has an error of 2.27 cm, with a ground resolution of 87.1 cm/pixel. The flight altitude for the first area was 70 metres, while for the second area, it was 80 metres.

Sample collection, pathogens isolation and characterization

During 2022, twenty-five symptomatic paulownia trees were selected and labelled and from each tree 500 g of rhizosphere samples and root tissues were collected around the collar. At the same time, bark samples were excised from stems showing sunken cankers and inner bark necrotic lesions. From three additional plants, showing aerial and extensive sunken cankers, two stems were cut and collected. The samples were analysed in the laboratory to determine the causal agents involved in the symptoms observed. In particular, 300 g of soil samples were placed in plastic containers with about 2 L of distilled water. A few hours later, when the soil particles became sediment, young leaves of *Q. ilex* were added on the water surface and left at 18–20°C for 3–5 days. The leaves showing dark-brown necrotic spots were placed in Petri dishes containing potato dextrose agar (PDA, Oxoid Ltd., Basingstoke, UK) amended with 100 mL L^{-1} of carrot juice, 0.013 g L^{-1} of pimaricin and 0.05 g L^{-1} of hymexazol (PDA+) (Linaldeddu *et al.*, 2020). For root samples, the isolation was performed directly from the necrotic tissues, removing small inner bark fragments and placing them both in PDA + and PDA amended with ampicillin (150 mg L^{-1}) and streptomycin (150 mg L^{-1}).

Morphological identification of the colonies obtained in pure culture was performed according to the colony appearance on PDA or carrot agar (CA) after 7 days at 20°C in the dark, and biometric data of mycelium and reproductive structures (conidia and sporangia) visualized under light microscope. All isolates were stored in PDA tubes at 5°C in the collection of the Department of Agriculture, Food and Environmental Science (Marche Polytechnic University, Ancona, Italy) and sterile paraffin oil in the culture collection of the Dipartimento Territorio e Sistemi Agro-Forestali (Università degli Studi di Padova, Italy).

Molecular analysis and phylogeny

The identity of all the isolates was inferred by molecular tools. Genomic DNA from mycelium of pure culture was extracted according to Bregant *et al.* (2020) and the ITS region was amplified and sequenced for all isolates with the primers ITS1 and ITS4 (White *et al.*, 1990) according to Linaldeddu *et al.* (2023). In addition, the primer-pairs TUBUF2/TUBUR1 (Kroon *et al.*, 2004) were used to amplify and sequence a portions of the β-tubulin (Btub) region of a representative set of *Phytophthora* isolates; whereas for *Botryosphaeriaceae* isolates a portion of the translation elongation factor 1 alpha gene (*tef1*-α) was amplified and sequenced with primers EF446f and EF1035r (Inderbitzin *et al.*, 2010).

Amplicons were purified, sequenced by BMR Genomics (Padova) and then edited with BioEdit software. The *consensus* sequences were compared with reference sequences (ex-type culture or representative strains) available in GenBank. The species was assigned when the nucleotide identity was 100% with sequences of ex-type culture. ITS and *tef1*-α sequences of two representative isolates of *Botryosphaeria dothidea* (accession numbers: OR551463, OR784637) and *Macrophomina phaseolina* (OR551464, OR784638) as well as ITS and Btub sequences of *Phytophthora citrophthora* isolate (OR551465, OR784639), *P. erythroseptica* (OR551466, OR784640) and *P. pseudocryptogea* (OR551467, OR784641) were deposited at GenBank.

In addition, a multigene phylogeny based on concatenated ITS and Btub sequences for *Phytophthora* spp. and ITS and *tef1-α* sequences for *Botryosphaeriaceae* was performed. Sequences were aligned with ClustalX v. 1.83 (Thompson *et al.*, 1997), using the parameters reported by Bregant *et al.* (2020). Phylogenetic reconstructions were performed with MEGA-X 10.1.8, including all gaps in the analyses. The best model of DNA sequence evolution was determined automatically by the software (Kumar *et al.*, 2018). Maximum likelihood (ML) analysis was performed with a neighbourjoining (NJ) starting tree generated by the software.

Pathogenicity tests

Given that all species isolated were not reported on paulownia, Koch's postulates were satisfied. Three *Phytophthora* species and two fungal species belonging to *Botryosphaeriaceae* were inoculated at the collar of potted 1-year-old rooted cuttings in June 2023, when they were highly vigorous and 60 cm tall according to Linaldeddu *et al.* (2023). The plants inoculated with a representative isolate of each species (ten plants per patho-

gen) were maintained in controlled conditions at around 22°C for 30 days. At the end of the experimental period, the presence of internal (necrotic lesion) and external (wilting and exudates) disease symptoms as well as the impact on the root systems was recorded. The size of the necrotic lesions was estimated by removing the outer bark. Finally, re-isolation of the pathogens was performed taking five pieces of symptomatic inner bark tissue and transferring them onto PDA+ (for *Phytophthora*) and PDA (for *Botryosphaeriaceae*).

Results of the pathogenicity test were checked for normality, then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined by Fisher's Least Significant Difference (LSD) test ($P = 0.05$) using XLSTAT 2008 software (Addinsoft, Paris, France).

RESULTS AND DISCUSSION

Field surveys conducted in both plantations over a two-year period, showed a high percentage of symptomatic plants. The first symptoms of vegetation suffering, characterized by yellowing and small sized leaves,

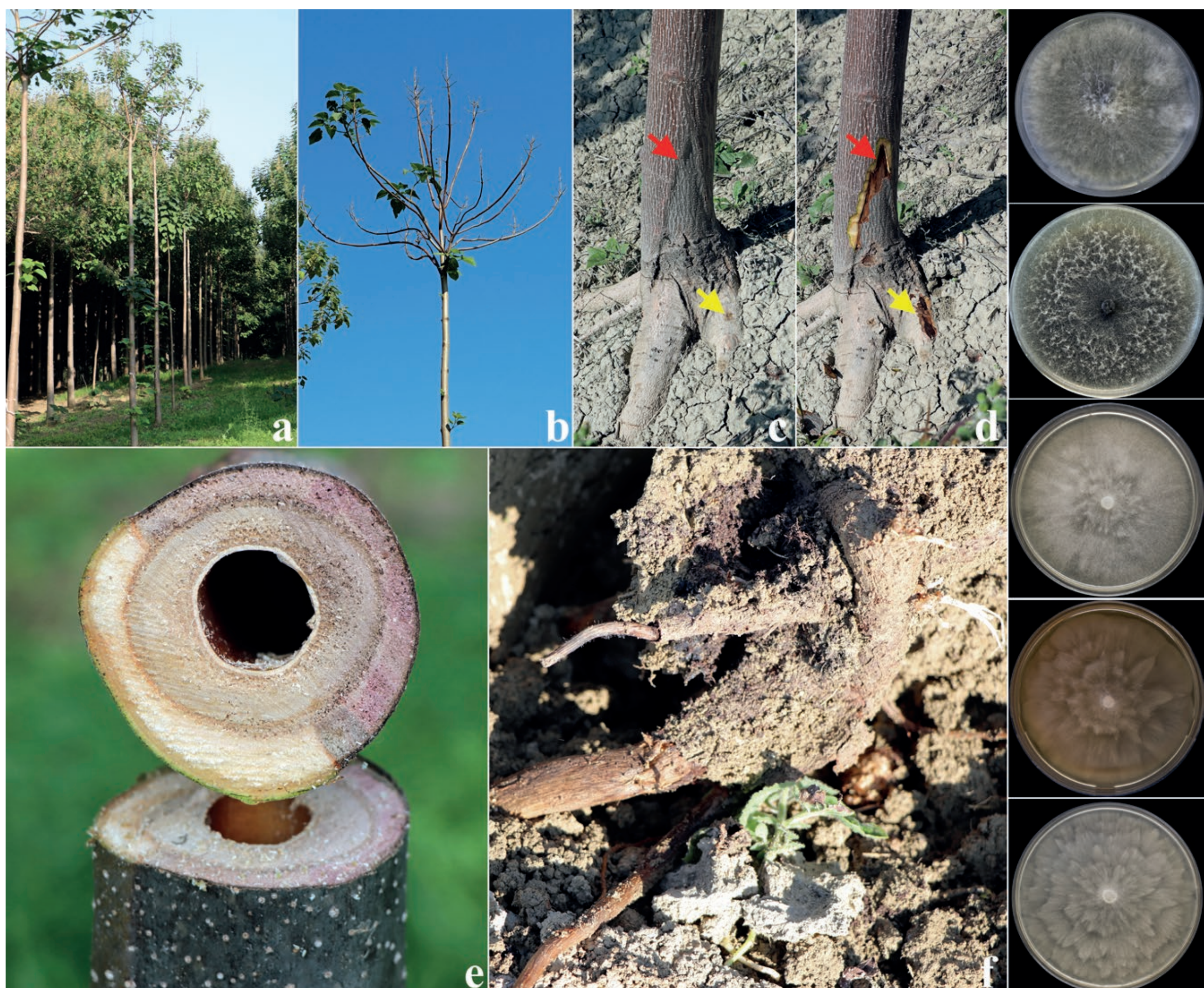

Figure 1. Overview of symptoms detected on the paulownia plants monitored in the study: extensive canopy dieback (A and B); tree showing a sunken canker at the collar caused by *Macrophomina phaseolina* (red arrow) and a *Phytophthora* bleeding canker on the main root caused by *Phytophthora pseudocryptogea* (yellow arrow) (C), particular of the internal (inner bark) necrotic lesion on the same tree (D), sunken canker in cross section (E), typical *Phytophthora* root rot symptoms (F). From top to bottom, colony morphology of *Botryosphaeria dothidea*, *Macrophomina phaseolina, Phytophthora citrophthora*, *Phytophthora erythroseptica* and *Phytophthora pseudocryptogea* after 7 days growth at 20 °C on PDA (*Botryosphaeriaceae*) and CA (*Phytophthora*) in the dark.

Table 1. Number of paulownia trees, cultivated in Site 1 and 2, showing different degrees of symptom severity in July 2022 and 2023.

Symptom severity	Site 1		Site 2	
	2022	2023	2022	2023
0	1668	1620	484	13
1	17	45	256	357
2	45	62	52	243
3	60	92	83	135
4	25	56	125	252
Total	1875		1000	

as well as percentage of canopy desiccation around 30%, were recorded in spring (Figure 1). The phytosanitary situation drastically declined during July, when canopy dieback was very frequent, with a percentage of desiccation around 30–50%, and dead plants.

In Site 1, the disease assessment performed during July 2022 and 2023 allowed an increment of symptomatic plants from 147 to 255 to be detected, as well as of dead plants. Most of the symptomatic plants showed an advanced status of canopy dieback (severity class 3) (Table 1).

In site 2, the phytosanitary status was completely deteriorated. More than 50% of plants were symptomatic in 2022 and in 2023 only 13 plants were without symptoms. In Site 2 in 2023 about 600 plants were evaluated with symptom severity in class 1 and 2, characterized by canopy dieback corresponding to <30% and between 30 and 50%, and 252 dead plants, double the number with respect to 2022 (Table 1). Among the 250 trees monitored, 173 showed both *Phytophthora* (bleeding) and *Botryosphaeriaceae* (sunken) cankers, 9 only *Phytophthora* bleeding cankers and 4 only *Botryosphaeriaceae* cankers, whereas 60 trees were dead (Figure 1).

By monitoring and disease assessing using aerial remote sensing (RS) in 2023, in Site 1 it was clear that the disease focus was not strictly related to dead plants, but there are areas, located near them, in which the pale green canopy captured during the flight, well correlated with the data collected in the field (Figure 2A). In Site 2, the aerial picture confirmed the dramatic phytosanitary *status*, extending to whole plantation (Figure 2B).

From 25 samples collected in the two paulownia plantations, we were able to isolate 22 *Phytophthora* colonies, of which 3 were obtained directly from necrotic canker tissues, and 19 indirectly from rhizosphere and root samples using the baiting technique. The morphological identification corroborated by molecular data, based on the sequences of the ITS and Btub regions, allowed three different *Phytophthora* species to be defined, namely *P. citrophthora* (8 isolates), *P. erythroseptica* (3 isolates), *P. pseudocryptogea* (11 isolates) (Figure 1 and S1).

Figure 2. Disease assessing by aerial remote sensing (RS) using drones in 2023: orthomosaic images of Site 1 (A) and Site 2 (B), collected with the DJI Mavic 3M.

From 12 samples collected from stems showing aerial sunken canker, we were able to isolate 11 fungal colonies belonging to *Botryosphaeriaceae*. In particular, three isolates were identified as *Botryosphaeria dothidea* and 8 as *Macrophomina phaseolina* (Figure 1 and S2,3).

The five species used in the artificial inoculation showed to be pathogenic on paulownia. The average lesion size differed significantly according to the species, e.g., the lesions caused by *B. dothidea* (7.50 ± 0.46a cm; mean ± standard deviation) and *M. phaseolina* (7.45 ± 0.32a) were significantly bigger than those caused by *P. pseudocryptogea* (6.20 ± 0.18b), *P. erythroseptica* (4.51 ± 0.23c) and *P. citrophthora* (4.30 ± 0.29c).

The three *Phytophthora* species caused a range of both not specific symptoms such as yellowing, progressive dehydration and desiccation of leaves and specific symptoms such as inner bark necrosis on stem and root rot (Figure 3 e-j). The most severe symptoms were induced by *P. pseudocryptogea*, the necrotic lesion caused by this species expanded from the inoculation site to the root system. Less severe symptoms of decline were recorded for plants artificially inoculated with *P. erythroseptica*. The necrotic lesions caused by *P. citrophthora* were confined to inner bark tissues.

Macrophomina phaseolina and *B. dothidea* showed to be very aggressive on paulownia. The necrosis developed very quickly and progressively girdled the stem

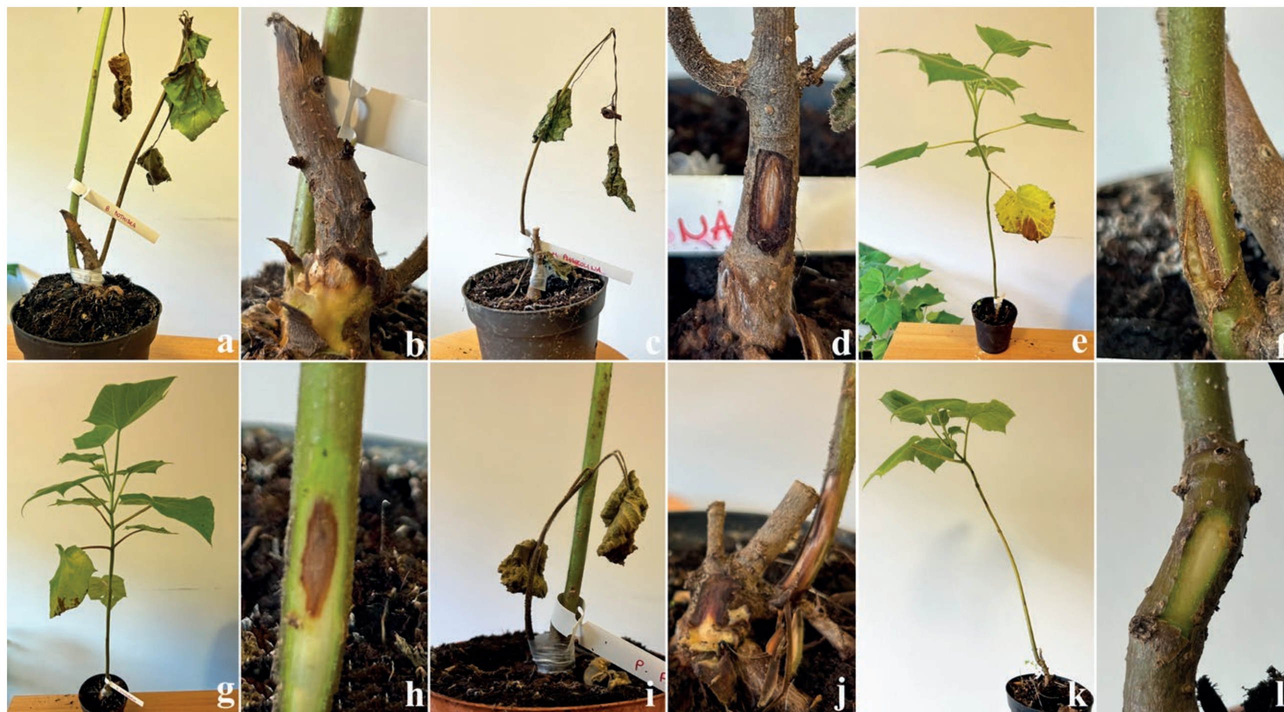

Figure 3. Artificial inoculation of *Botryosphaeria dothidea* (a,b), *Macrophomina phaseolina* (c,d), *Phytophthora citrophthora* (e,f), *P. erythroseptica* (g,h) and *P. pseudocryptogea* (i,j) on 1-year-old Paulownia plants in accordance with Koch's postulates. Control seedling (k,l).

causing wilting symptoms, with dead leaves remaining attached to the plant (Figure 3 a-d).

The symptoms induced by *Phytophthora* species, *M. phaseolina* and *B. dothidea* were identical to those observed in the two plantations of paulownia, except for exudates that have not been recorded on the young plants, artificially inoculated.

All five pathogens were successfully re-isolated from the margin on necrotic inner bark lesions of all seedlings, thus fulfilling Koch's postulates. All species are reported here for the first time as paulownia pathogens worldwide.

In conclusion, the findings obtained in this study allowed us to define the aetiology of the decline affecting paulownia trees in Italy, contributing to expand knowledge on the hosts range of some aggressive pathogens belonging to the genera *Botryosphaeria*, *Macrophomina* and *Phytophthora*. The co-occurrence of *Phytophthora* and *Botryosphaeriaceae* species was recently detected in several emerging diseases affecting forest trees and agriculture crops in Italy (Benigno *et al*., 2023; Linaldeddu *et al*., 2023). This complex aetiology indicates that multitrophic interactions are common in forest plantations and represent an important and concrete aspect of tree-pathogen relationships, providing a more realistic picture of the dynamics contributing to tree decline and mortality.

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Research Papers

Phytophthora spp. diversity in commercial nursery stocks shown through examination of plant health practices for growers and traders of ornamental plants

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Summary. Management of *Phytophthora* in commercial plant nurseries is important for biosecurity of traded plants, and monitoring of incidence of this important plant pathogen is a prerequisite to prevent its spread. Potted plants showing *Phytophthora* spp. symptoms, and nursery irrigation and runoff water, were sampled from a commercial and a non-commercial nursery in Tuscany, Italy. The samples were processed to detect *Phytophthora* spp., using baiting, and molecular identification of obtained isolates. High *Phytophthora* incidence was shown in the commercial nursery. Twelve *Phytophthora* spp. were isolated from potted plants or nursery runoff water. Individual symptomatic potted plants were infected with up to four pathogenic *Phytophthora* spp. The water sampled from nursery drainage canals had the greatest *Phytophthora* species diversity, with less diversity in 'flow-through' water samples (irrigation water percolated through potted plants) and samples from water puddles inside the nurseries. This study showed high incidence of *Phytophthora* in the commercial nursery, and associated risk of spread of these pathogens within and outside nursery operations. Lack of appropriate disease management probably increases occurrence of these pathogens.

Keywords. Oomycetes spread, biological hazard, potted plants health, stakeholder involvement, risks warning.

INTRODUCTION

Phytophthora spp. are plant-damaging oomycetes (*Peronosporales*) that can cause significant economic losses in many different crops. From approx. 500 estimated species (Yang *et al.*, 2017). More than 200 *Phytophthora* type species have been described (Abad *et al.*, 2023). Most of these taxa are poten-

tially invasive and lethal pathogens of woody plants (Brasier, 1999), that have been directly responsible for ecological, economic and social impacts on a continental scale during the past 150 years (Brasier *et al*., 2022).

Phytophthora is strictly linked to soil for dispersal, is well-adapted to living in water, and spreads from plant to plant via motile zoospores (Erwin and Ribeiro, 1996). Many species can survive in soil as chlamydospores, under unfavourable conditions and for long periods (Hwang, 1978; Fichtner *et al*., 2007; Shishkoff, 2007). Persistent high humidity, close proximity to potential host species, movement of plant growth media and irrigation water, general lack of sterilization steps in plant propagation, and use of external or imported plant propagation material, make commercial nurseries the sites of introduction, survival and spread of many *Phytophthora* spp. (Themann *et al*., 2002; Moralejo *et al*., 2009; Migliorini *et al*., 2015; Jung *et al*., 2016). Such conditions explain the many destructive outbreaks of these pathogens that have occurred in nurseries during the last decades (Brasier *et al*., 2022, and other publications cited in the present paper). Nurseries that produce plants in pots are therefore responsible for spreading of *Phytophthora* spp. due to the significant presence of *Phytophthora* inoculum in soil and roots of the final products, which are sold as asymptomatic plants (Migliorini *et al*., 2015).

For these reasons and during the last decade, large scale investigations have aimed to characterize *Phytophthora* diversity in plant nurseries, and have been implemented at national level, with the scope to identify the greatest phytosanitary risks in individual nurseries and in the production links between nurseries. Examples are the outcomes obtained in Oregon, United States of America, by Parke *et al.* (2014), and by Schiffer-Forsyth *et al.* (2023) in the United Kingdom as part of the PHYTO-THREATS project (Green *et al*., 2020, 2021). Through results of extensive diagnostic services based on molecular techniques, both of these studies provided foundations for implementing systems approaches in nursery production, by providing information on *Phytophthora* spp. presence and abundance at critical control points, and outlining best disease management practices.

The present research has been part of the EUPHRESCO project 'ID-PHYT' ("EUPHRESCO 'ID-PHYT-Early detection of *Phytophthora* in EU and third country nurseries and traded plants').

The objective of this study was to characterize *Phytophthora* spp. in a commercially active retail nursery which had robust production and frequent exchanges of potted woody plants, and in a non-commercial nursery with minimal entry and exit of potted plants. These two nurseries were situated in the same geographic area. The results of this study have been shared with the project partner, and have been used within this study to enhance *Phytophthora* sampling for refinement of best management practices in productive ornamental nurseries.

MATERIALS AND METHODS

Sample collection

The two potted-plant nurseries selected for this study were in Northern Tuscany, Italy, within the peri-urban areas of Florence (nursery 1) and Pistoia (nursery 2). Nursery 1 (N1) was a non-commercial, research nursery, while nursery 2 (N2) was a commercial retail nursery associated with international trading of potted plants.

Following the 'ID-PHYT' protocol, selection of sample types aimed to maximize taxonomic characterization of *Phytophthora* spp. Care was taken to extend detection to all potential inoculum sources within the two nurseries. Samples analysed in consisted of: i) potted plants, ii) potted plant 'flow-through water' (see below), and iii) water from the irrigation systems. Sample types slightly differed between N1 and N2. Plant samples selected in N1 and N2 were of different species. Water samples from N1 were collected from the irrigation pipe system and the irrigation pond. Water samples from N2 were collected from irrigation pipes, nursery runoff water and puddled water (Puddles) (Table 1). Sampling occurred in May 2021, according to criteria outlined below.

Potted plants

Two plants per species, showing dieback symptoms (leaf discolouration, and/or leaf spotting, poor foliage development) were selected, and then processed for the 'flow-through" procedure (Flow-through water, see below). They were then brought to a laboratory where the associated potting soil (Potting soil), consisting of soil and roots, was processed using baiting for isolation of oomycetes.

Flow-through water

Potted plants when still in the nursery were placed in sterile trays, and were irrigated with local irrigation water to reach the 10–20% of pot water holding capacity for 20 min, to stimulate release of Potting soil Oomycete inoculum into the trays. Water from the trays was then

Table 1. Sources of the samples collected form two nurseries. The samples consisted of potted plants, potted plant 'flow-through water' and water from the irrigation systems. Plant samples from the two nurseries of different species. Water samples from N1 were collected from the irrigation pipe and the irrigation pond, and samples from N2 were from the irrigation pipe, runoff nursery water, and puddled water present in the nursery.

Sample source	Nursery		Potting Soil	Water	
	1	2		Irrigation system	Flow-through
Irrigation pond	/			/	
Irrigation pipe	/			/	
Cupressus sempervirens	/		/		/
Fagus sylvatica	/		/		/
Ilex aquifolium	/		/		/
Myrtus communis	/		/		/
Pinus nigra	/		/		/
Ulmus minor	/		/		/
Viburnum tinus	/		/		/
Irrigation pipe		/		/	
Runoff water		/		/	
Puddles		/		/	
Magnolia grandiflora		/	/		/
Choisya ternata		/	/		/
Choisya ternata 'Aztec Pearl'		/	/		/
Ceanothus concha		/	/		/
Elaeagnus angustifolia		/	/		/

collected in sterile tanks and processed in the laboratory for oomycetes isolations.

Water

Water from the nursery irrigation systems, including an irrigation pond (N1), water from irrigation pipes (N1 and N2), water from small puddles on the dirt roads of N2, and runoff water of N2, was collected in previously sterilized water tanks and processed for oomycetes isolations.

Sample processing

Baiting

All samples, including Potting soil, Flow-through and Puddles water, and water from irrigation pipes and pond, were immediately processed for isolation of oomycetes in the laboratory, using the baiting technique outlined in Figure 1. The analysis was conducted according to 'Baiting Method 1' described by Burgess *et al.*, 2021, with the following exceptions: each sample was analysed in duplicate (two containers each); containers were 280 × 190 × 140 mm deep, filled with a final volume of 3 L of distilled water. Baits used were young leaves of *Hedera* sp. or young leaves and rose petals of *Quercus ilex* (Figure 1); all isolation culture plates were incubated at 20°C in the dark, and checked daily for growth of oomycetes.

Figure 1. Baiting of water and potted plant samples. Containers were 280 × 190 × 140 mm deep, and each contained 3 L of distilled water. Baits used were young leaves of *Hedera* sp. and *Quercus ilex*, and rose petals.

DNA sequencing

Cultures were transferred onto '½ PDA' medium plates (19.5 g L^{-1} of Potato dextrose agar, 7.5 g L^{-1} of Agar, 1 L of deionized water). Aerial hyphae (ca. 80 mg) were scraped from the surface of each culture, and then ground in a 2 mL capacity microfuge tube with two tungsten beads (3 mm) (Qiagen) and 400 µL of Buffer P1 (EZNA Plant DNA Kit, Omega Bio-tek), using a Mixer Mill 300 (Qiagen) set for 2 min at 20 Hz. DNA was extracted from all samples using the EZNA Plant DNA Kit (Omega Bio-tek), following the manufacturer's instructions. The DNA concentrations were measured using a Nanodrop ND1000 spectrophotometer (NanoDrop Technologies). For phylogenetic analyses, the internal transcribed spacer ITS regions (including spacers ITS1 and ITS2 and the 5.8S gene of the rDNA) were amplified using the primers ITS6 and ITS4 (White *et al.*, 1990; Cooke *et al.*, 2000), following the protocol by Migliorini *et al.* (2020). PCR amplicons were purified with a miPCR Purification Kit (Metabion International), and were sequenced in one direction by Macrogen (Seoul,

South Korea). The qualities of amplified nucleotide sequences were checked with the Geneious ver. R10 software package (Biomatters; https://www.geneious.com/).

Phylogenetic analyses

BLAST searches of the generated sequences were carried out using the NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), to identify the most closely related sequences. Isolate sequences of *Pythiaceae* were compared to those of known *Pythium* and *Phytopythium* spp. obtained from GenBank. The ITS sequences of *Phytopythium* and *Pythium* were from *Pythium kashmirense* (HQ643671), *Phytopythium vexans* (HQ643400) (Robideau *et al*., 2011) or *Phytopythium paucipapillatum (*KX372749, Crous *et al*., 2020). BLAST searches of the generated ITS gene sequences of *Phytophthora* were carried out using a custom database to identify the most closely related sequences. The *Phytopthtora* database sequences were from the type isolates found on IDPhy (Abad *et al*., 2023). The BLAST search of *Phytophthora* and the subsequent phylogenetic analysis, and the analyses of *Pythium* and *Phytopythium*, were conducted at Geneious. Sequences were aligned using the MAFFT alignment within Geneious, using the default parameters. Phylogenetic trees were constructed in Geneious Tree Builder using the Neighbour-Joining Method (Genetic Distance Model: HKY). Bootstrap was selected as Resampling method (2000 Number of Replicates). Gaps were treated as missing data.

RESULTS

Thirty-eight isolates were obtained in this study, of which four were from N1 (Florence) and 34 were from N2 (Pistoia). Twenty-seven of the isolates were from water samples, including 12 from Run-off, 13 from Flow-through, two from Puddles, and one from the irrigation pond. Ten isolates were obtained from Potting soil. The ITS sequences were sufficient to identify each isolate. The 15 detected species were: *Phytophthora acerina, Phytophthora cactorum, Phytophthora cambivora, Phytophthora chlamydospora, Phytophthora cinnamomi, Phytophthora gonapodyides, Phytophthora hydropatica, Phytophthora lacustris, Phytophthora multivora, Phytophthora nicotianae, Phytophthora plurivora, Phytophthora pseudocryptogea* (Figure S1), *Pythium kashmirense, Phytopythium paucipapillatum, Phytopythium vexans* (Figure S2). Three isolates of *Pythium* and *Phytopythium,* and one of *Phytophthora gonapodydes* were obtained from N1. All the other *Phytophthora* species, including *P. gonapodyides,* were isolated from N2 (Table S1). Sequences were deposited in the GenBank (Table S1).

Phytophthora *taxa*

Twelve *Phytophthora* spp. were isolated from N2, and one isolate of *P. gonapodydes* was obtained from N1 (Table 2). Seven different *Phytophthora* spp. were detected in Run-off water samples, four each in Flow-through and Potting soil samples, and one was detected in Puddles samples, while none were detected in the irrigation pond (Table 2, Figure 2). Several *Phytophthora* species were detected from N2, from different matrices and/or plant species: *P. cinnamomi* from Flow-through of *Magnolia grandiflora* and *Ceanothus concha*, and from Potting soil of *C. concha* and *Elaeagnus angustifolia*; *P. gonapodyides* from Run-off water and Puddles; *P. nicotianae* from Potting soil of *Choisya ternata* 'Aztec Pearl', and Flow-through of *C. ternata* and *C. concha; P. plurivora* from Flow-through of *M. grandiflora* and *C. ternata* 'Aztec Pearl', and from Run-off water (Table 2). *Ceanothus concha* was the plant species from which the largest number of *Phytophthora* species were isolated (*P. cinnamomi* and *P. nicotianae* from Flow-through and *P. cactorum*, *P. cinnamomi* and *P. multivora* from Potting

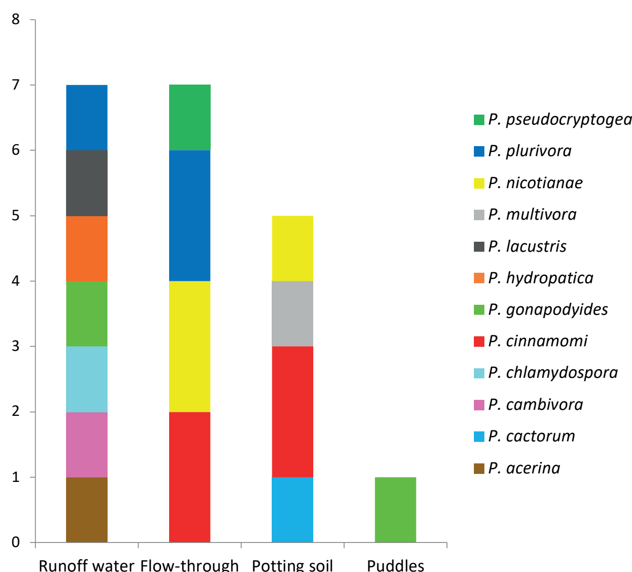

Figure 2. Distributions of the twelve *Phytophthora* spp. isolated in this study, across the four isolation matrices (Runoff water, Flow-through, Potting soil or Puddles). Each rectangular unit represents one detection of each pathogen per matrix per plant species. The double rectangular units for *P. cinnamomi*, *P. nicotianae* and *P. plurivora* indicate that these organisms were detected from the same isolation matrix in two different plant species (see Table 2 for taxonomic details).

Table 2. List of *Phytophthora* species obtained in this study. The table indicates the nursery of provenance, if the pathogen was isolated from one of the sampling categories, including Runoff water, Flow-through, Puddles, irrigation pond or from Potting soil. Plant species from which Flow-through and Potting soil isolates were obtained are also indicated. Numbers of total species and total species from each isolation matrix in each plant species per each matrix are also summarized.

Phytophthora species	Nursery		Plant species	Isolation matrix				
				Water				Potting soil
	1	2		Runoff water	Flow-through	Puddles	Irrigation pond	
P. acerina		/		/				
P. cactorum		/	*Ceanothus concha*					/
P. cambivora		/		/				
P. chlamydospora		/		/				
P. cinnamomi		/	*Magnolia grandiflora*		/			
		/	*Ceanothus concha*					/
		/	*Ceanothus concha*		/			
		/	*Elaeagnus angustifolia*					/
P. gonapodyides		/		/				
		/				/		
P. hydropatica		/		/				
P. lacustris		/		/				
P. multivora		/	*Ceanothus concha*					/
P. nicotianae	/		*Cupressus sempervirens*					/
		/	*Choisya ternata* 'Aztec Pearl'					/
		/	*Choisya ternata*		/			
		/	*Ceanothus concha*		/			
P. plurivora		/	*Magnolia grandiflora*		/			
		/		/				
		/	*Choisya ternata* 'Aztec Pearl'		/			
P. pseudocryptogea		/	*Choisya ternata* 'Aztec Pearl'		/			
Species per isolation matrix per plant species				7	7	1	0	6
Total species	1	12		7	4	1	0	4

soil), followed by *C. ternata* 'Aztec Pearl' (*P. plurivora* and *P. pseudocryptogea* from Flow-through and *P. nicotianae* from Potting soil), *M. grandiflora* (P. *cinnamomi* and *P. plurivora* from Flow-through), *E. angustifolia* (*P. cinnamomi* from Potting soil) and *C. ternata* (*P. nicotianae* from Flow-through) (Figure 3).

DISCUSSION

Several cases of spread of different *Phytophthora* species have been reported from non-commercial plant restoration nurseries into wild areas, where these oomycetes had not been previously detected (Rooney-Latham *et al.*, 2015, 2019). In the present study, however, in the non-commercial nursery (N1) only one *Phytophthora* sp. was detected, as a single isolate. In contrast, several *Phytophthora* spp. known as pathogens of different host plant species, and from different isolation matrixes, were found in the commercial nursery (N2), including 12 species from six *Phytophthora* clades.

Several important pathogens, including *P. cactorum*, *P cinnamomi*, *P. pseudocryptogea*, *P. nicotianae* and *P. plurivora*, were isolated from one location. All of these *Phytophthora* spp. are classified as polyphagous species, which are well-adapted to nurseries, forestry, and agricultural environments (Jung *et al.*, 2018). Within this group, *P. cinnamomi* is particularly important. This organism is one of the most devastating plant pathogens, in terms of geographic distribution and host range. It has been listed as one of the 100 worst invasive species (Burgess *et al.*, 2017), and is well-known as the cause of large-scale dieback of *Eucalyptus* (jarrah dieback) in Australian forests (Dell and Malajczuk, 1989) and as a cause of oak decline in the Iberian Peninsula (Brasier *et al.*, 1993).

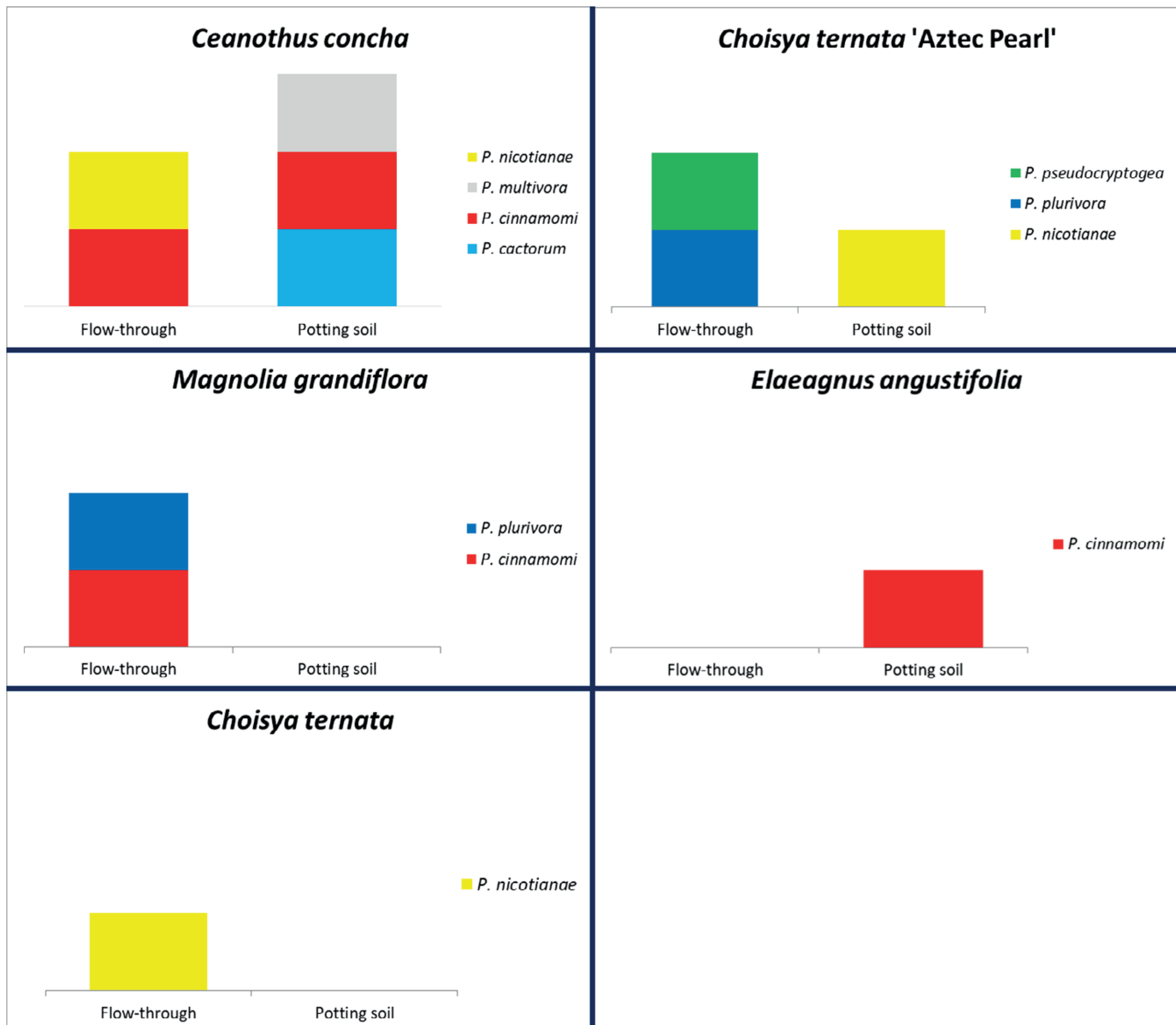

Figure 3. *Phytophthora* spp. isolated in this study from five plant hosts. The two columns in each histogram indicate the isolate sources (Flow-through or Potting soil; see text).

During the present study, *P. cinnamomi* was detected from three plant species, and from potting soil and flow-through water, underlining the potential spread of viable inoculum across a nursery. *Phytophthora nicotianae* was similar, being found on three host species and in the same isolation matrices. Like *P. cinnamomi*, *P. nicotianae* is a severe disease agent of many plant taxa, but apart from ornamentals and citrus trees, it is not responsible for dieback diseases of woody plants in the wild (Brasier *et al.*, 2022). *Phytophthora cactorum*, *P. pseudocryptogea*, and *P. plurivora* are notorious root and collar rot disease agents on many hosts, but while *P. cactorum and P. pseudocryptogea* have broad host ranges including herbaceous and crop species (Hudler, 2013; Delshad *et al.*, 2020), *P. plurivora* is mainly a woody host pathogen, both in woodland and on ornamentals (Jung and Burgess, 2009).

Phytophthora acerina, *P. cambivora* and *P. multivora* are aggressive woody plant pathogens that were found during the present survey. *Phytophthora acerina* was first reported on *Acer pseudoplatanus* and olive trees in northern Italy, and recently on *Metasequoia glyptostroboides* in China (Liu *et al.*, 2022) and walnut trees in California (Forbes *et al.*, 2019). *Phytophthora cambivora* has been frequently reported as the cause of ink disease of chestnut trees in southern and eastern Europe

(Vettraino *et al.*, 2005; Černý *et al.*, 2008), but this is also a species with broad international distribution and associated with declining trees. *Phytophthora multivora* is known as a dieback and bleeding canker agent in forest (Scott *et al.*, 2009, 2012) and urban trees of Western Australia (Barber *et al.*, 2013), where it has been demonstrated to be highly pathogenic on multiple native plants (Migliorini *et al.*, 2019). This species is now considered a significant pathogen with a wide host range and broad international distribution in nurseries, urban environments and natural ecosystems, and has been widely detected, mainly in nurseries of woody plants (Migliorini *et al.*, 2019; other reports cited elsewhere in this paper).

The other *Phytophthora* spp. detected in this survey included aquatic species that are common in nurseries but have not been associated with severe pathogenicity traits. These included *P. gonapodyides*, *P. lacustris*, *P. chlamydospora* from clade 6, and *P. hydropatica* from clade 9.

Other species of *Pythiaceae* were also detected in the present study. These belong to genera known for their pathogenicity on woody plants. However, these organisms are secondary concerns in mature potted plants cultivated in ornamental nurseries, as they are primarily damping-off agents affecting young hosts during the seedling stages. *Pythium kashmiriense* and *Phytopythium paucipapillatum* are rare soilborne species, which have been detected only once, respectively, in Europe (Benavent-Celma *et al.*, 2021) and South Africa (Crous *et al.*, 2020). *Phytopythium vexans* has aggressiveness and dissemination capabilities that are similar to some of the most pathogenic *Phytophthora* spp. isolated in the present study, although this pathogen does not exhibit the same levels of invasiveness in forests and natural environments (Panth *et al.*, 2021). Notably, *P. vexans* was the only relevant pathogen obtained in the non-commercial nursery.

The outcomes of this research indicate that the different plant production procedures used in two potted-plant nurseries may have determined their levels of biosecurity, emphasising that the implementation of effective management practices should be a priority in commercial nurseries. Both N1 and N2, the first with little presence of *Phytophthora*, the second with abundant *Phytophthora* spp. associated with all the different analysed sample types, did not utilize any biosecurity practises, such as filtering of irrigation water prior to use, cultivation of potted plants on benches, and use of pre-sterilized potting soil. It is probable that the difference in production procedures led to the difference in pathogen abundance between the two nurseries, both in pathogen taxa and their spatial distributions. The production techniques in N2, which did not differ from those of most of the retail nurseries located in the same area, were characterised by the constant input of propagation material from other producers. This practice is known to be linked to high biological risks, due to the potential abundance of pathogens (Ghelardini *et al.*, 2016; Eschen *et al.*, 2017). The non-application of simple, effective safety practices encourages spread and persistence of all newly introduced *Phytophthora* species within nurseries and results in losses to customers and to final recipients of plants, causing financial damage. The lack of biosecurity measures will lead to further ecological impacts where plants will be finally planted, both on large scales, through international trade in pot plants contaminated with pathogens, and locally, with spread of *Phytophthora* in areas neighbouring nurseries through contaminated irrigation water. This last aspect was documented in the present study, which detected up to seven species in N2 runoff water.

In conclusion, the results of this research demonstrate that non-adoption of internal prevention protocols aimed at systemic control of *Phytophthora* spp. in commercial nurseries can lead to severe economic losses. In this specific case, the N2 growers and traders were briefed on the necessary actions to be taken to implement a progressive process limiting infected propagation material and, consequently, producing and selling potted material that is not contaminated with *Phytophthora* spp.

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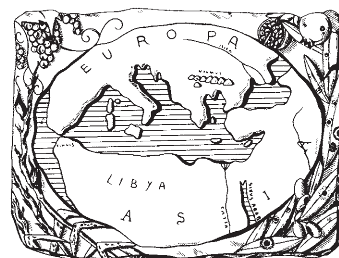

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