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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.

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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. petchii	**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.

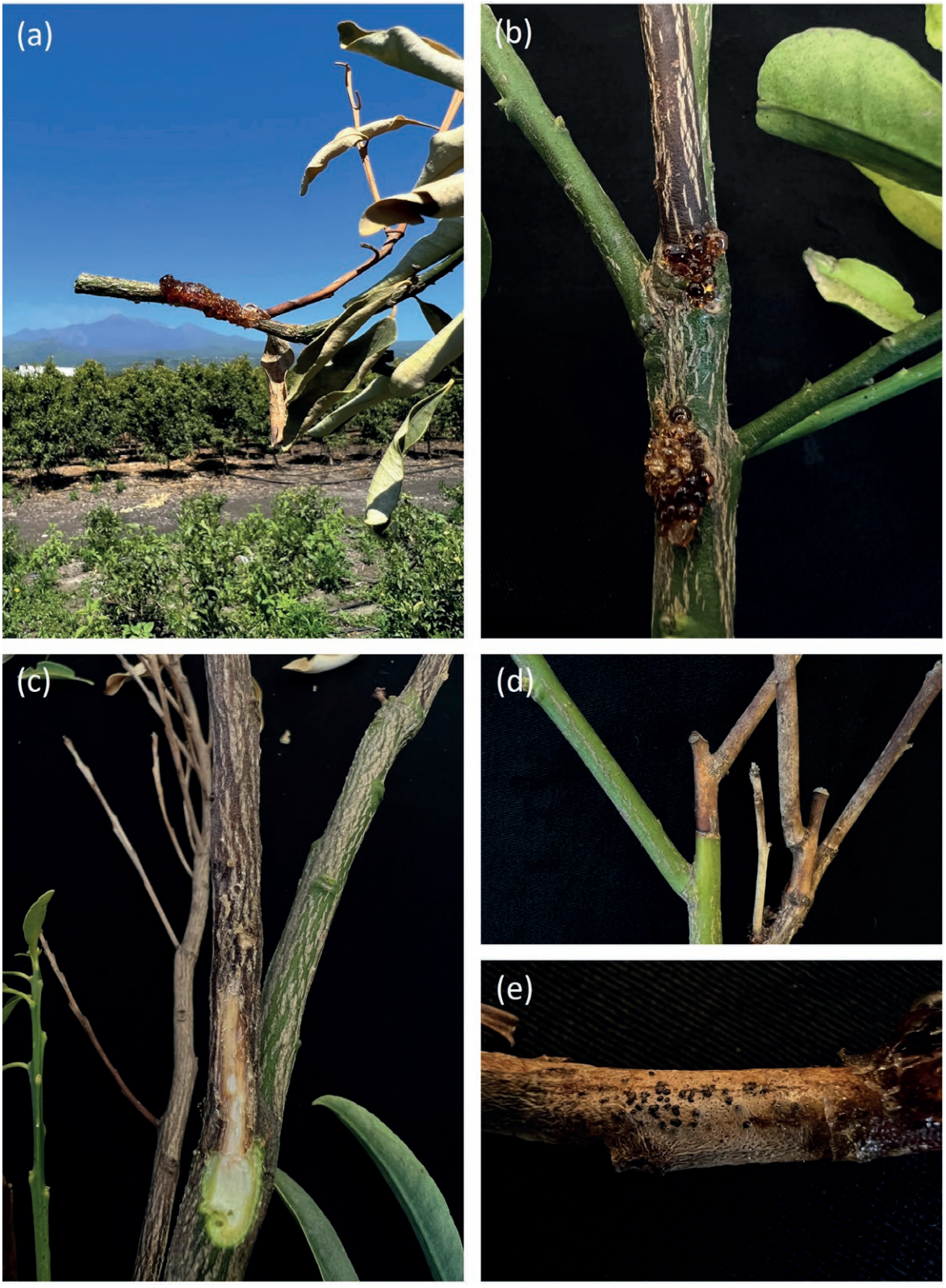

Figure 2. Symptoms of Colletotrichum dieback on kumquat (*Fortunella margarita*). a and b, gummosis and brown to chocolate-brown lesions on twigs; c, brown internal discolouration of twigs; d, detail of clearly-shape twig lesions; e, typical *Colletotrichum* acervuli on the surface of a dead host branch.

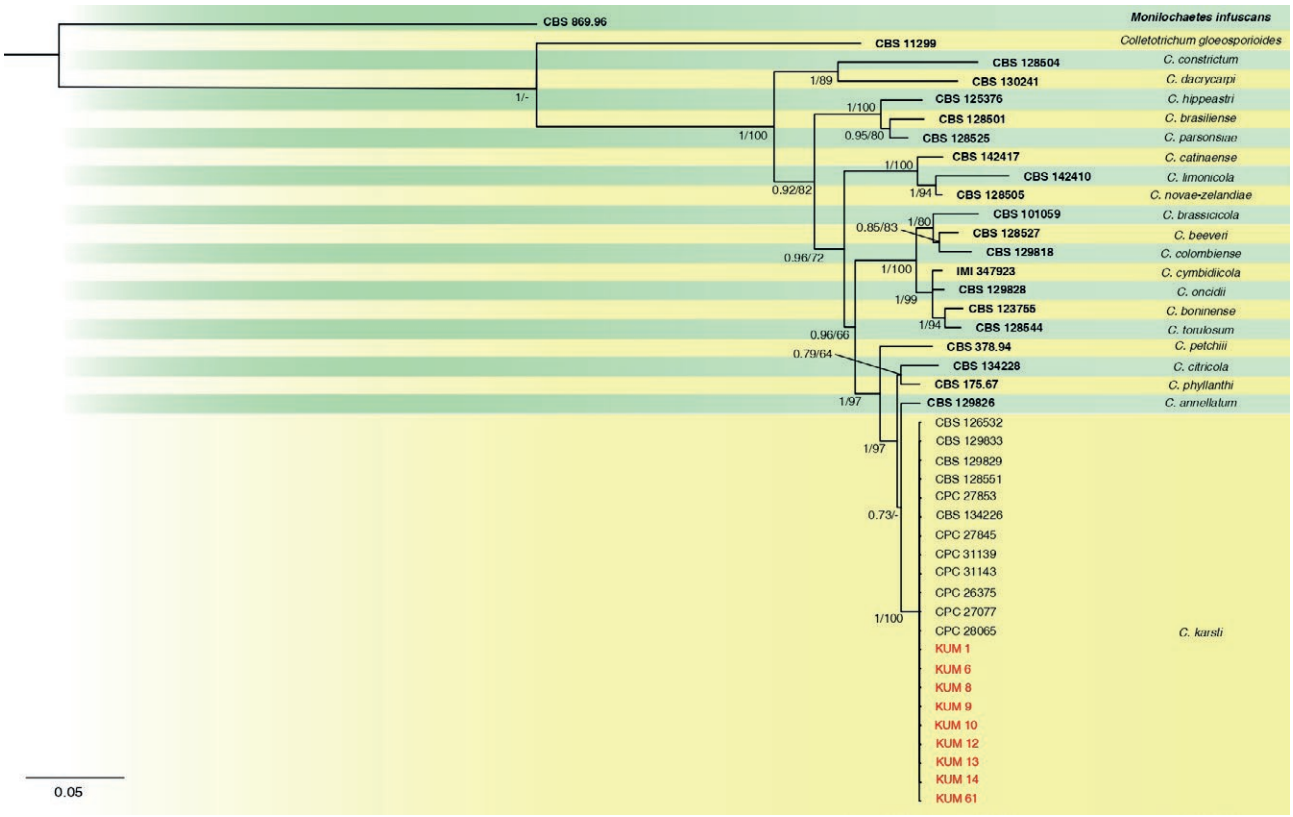

Figure 3. Consensus phylogram resulting from BI of the combined *gapdh*, *act* and *tub2* datasets. Bayesian posterior probability values and bootstrap support values are indicated at the nodes. The tree was rooted with *Monilochaetes infuscans* (CBS 869.96). The fungal isolates used in this study are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

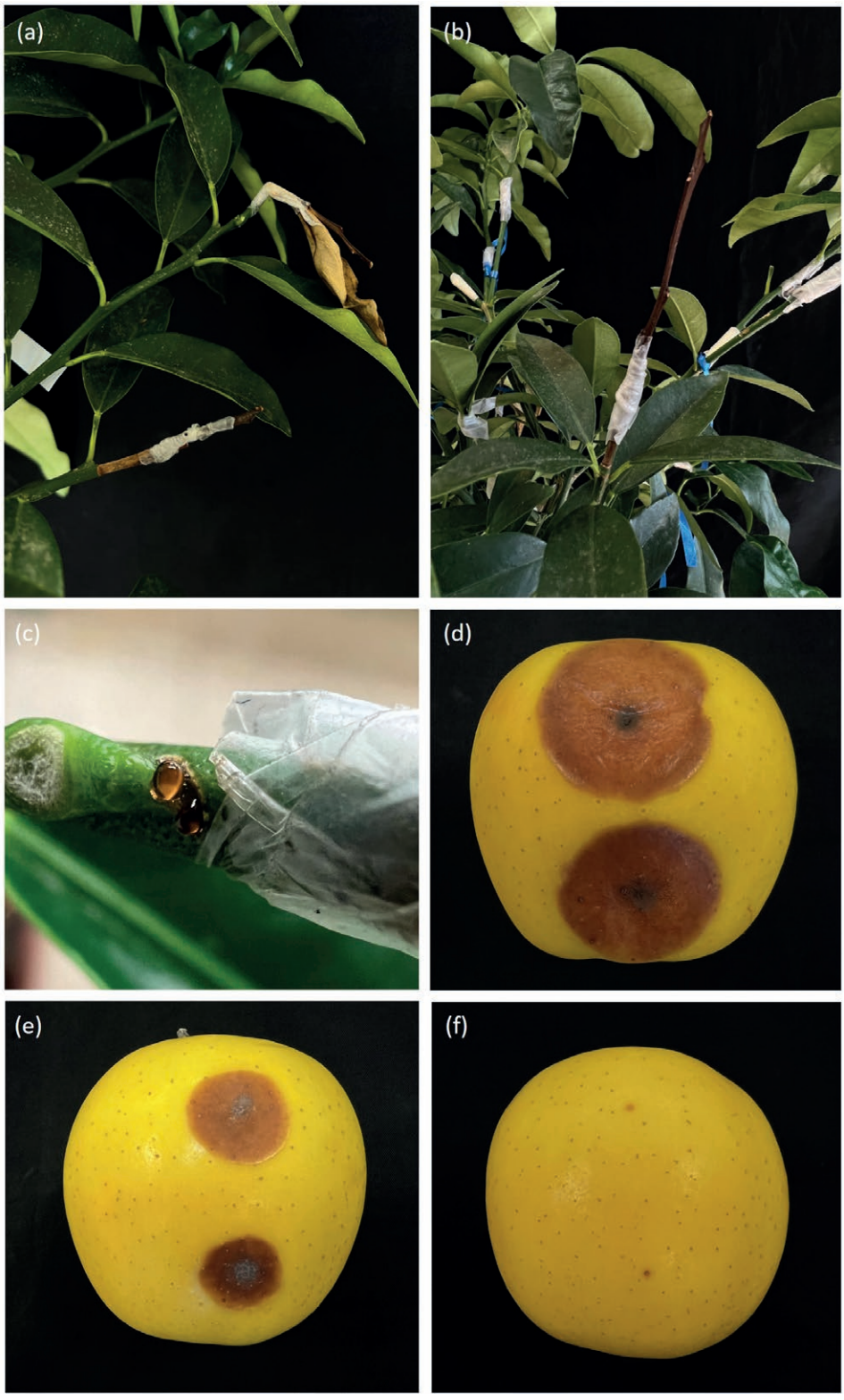

Figure 4. Pathogenicity and aggressivity tests. a and b, symptoms of twig dieback on 2-year-old kumquat plants (*Fortunella margarita*), 20 d after inoculation with mycelium plugs of *Colletotrichum karsti* isolate KUM6. c, detail of gummosis on a kumquat twig below the artificial inoculation point, 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.

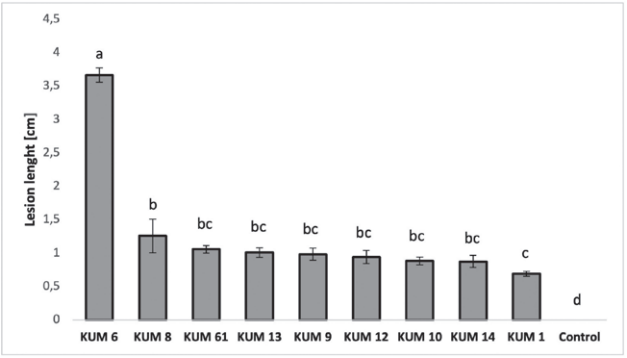

Figure 5. Mean lesion length (cm) resulting from inoculations with different *Colletotrichum karsti* isolates (KUM 6 to KUM 1) onto apple fruit of cultivar 'Golden Delicious' 7 d after inoculations. Different letters above the bars indicate statistically significant differences between the isolates, 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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