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Fungicide sensitivity assessment and susceptibility of newly bred olive lines, to improve anthracnose management in South Africa

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Summary. Olive anthracnose is an important disease in South Africa, which can result in high yield losses and reduced oil quality. This study aimed to identify the *Colletotrichum* species associated with olive anthracnose in South Africa, determine the *in vitro* efficacy of four fungicides against the fungi, and evaluate the susceptibility of newly bred olive lines to anthracnose. Olive fruit with typical anthracnose symptoms and twigs showing dieback were collected from olive farms in the Western Cape region. Isolations were made, and causal fungi were identified by amplification and sequencing of the internal transcribed spacers, partial β-tubulin and actin genes as well from their morphological characteristics. All isolates were identified as *Colletotrichum acutatum sensu stricto*. Fungicide sensitivity of mycelium growth and conidium germination were assessed for dodine, thiram and the mixtures of boscalid + pyraclostrobin and cyprodinil + fludioxinil. Mycelium growth and conidium germination of *C. acutatum* were strongly inhibited by boscalid + pyraclostrobin and cyprodinil + fludioxonil. The three most virulent *C. acutatum* isolates were assessed in a wounded fruit droplet inoculation trial on the olive cultivars Manzanilla (susceptible) and Mission (moderately susceptible). These isolates were also used to assess susceptibility of 18 newly bred olive lines and six reference cultivars. Unwounded fruit samples were dip inoculated with a mixture conidium suspension of the three *C. acutatum* isolates, and lesion development was recorded. Four potential oil lines and three green table olive lines showed resistance to the fungi. This study reports the first sequence confirmation of *C. acutatum* s.s. associated with anthracnose of olives in South Africa. Furthermore, the identification of the seven resistant olive lines enabled their selection for further evaluation and development. Fungicide sensitivity, together with newly bred line susceptibility results, will aid in development of integrated disease management for olive anthracnose.

Keywords. *Colletotrichum acutatum*, β-tubulin, actin, host resistance.

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INTRODUCTION

The South African olive industry is mainly based in the Western Cape region of that country, an area with typical Mediterranean climate which is ideal for growing olives (Costa, 1998). South Africa produces ~2.5 million litres of olive oil and 4000 tonnes of table olives from approx. 4000 ha annually (SA Olive Industry Association, 2024). Local production, although of high quality, is insufficient for local consumption, and a further 2.5 million litres of olive oil and 4000 tonnes of table olives are imported annually (SA Olive Industry Association, 2024). Most orchards are spaced conventionally and irrigated, while some super-intensive orchards have been recently established (C. Costa pers. comm.). The widely adapted *Olea europaea* subsp. *cuspidata* grows throughout Southern Africa and was initially used as a rootstock. Since the 1960s, commercial orchards consist of own-rooted trees of the cultivated European olive, *O. europaea* subspecies *europaea* (Mukadam, 2014). The cultivars grown commercially, in order of importance, include Frantoio, Mission, Coratina, Favalosa, Kalamata, Arbequina, Manzanilla, Picual, Koroneiki, Nocellara del Belice, Barouni and Leccino (C. Costa pers. comm.).

Anthracnose of olives is a severe disease which occurs in many olive-growing countries, including Argentina, Australia, Greece, Italy, Morocco, Portugal, South Africa, Spain, and Uruguay (Talhinhas *et al*., 2011; Cacciola *et al*., 2012; Msairi *et al*., 2017; Kolainis *et al*., 2020; Moreira *et al*., 2021). The most typical symptoms of this disease are observed on olive fruits after cuticle penetration by pathogens has occurred (Talhinhas *et al*., 2011; Cacciola *et al*., 2012). In moist conditions, the infected fruits show a growing brown rot lesions which ooze orange gelatinous matrices containing pathogen conidia, and in dry conditions, the affected fruit becomes mummified due to dehydration (Cacciola *et al*., 2012). Symptoms of leaf necrosis and branch dieback of infected olive trees have also been reported (Gorter, 1956; Talhinhas *et al*., 2011; Cacciola *et al*., 2012). Fruit yields for the table olive industry and oil quality for the olive oil industry are affected by this disease. Oil produced from infected fruit has a reddish colour, is off-flavour, of increased acidity, and has reduced contents of β-sitosterol, polyphenols, and α-tocopherol (Cacciola *et al*., 2012).

Yield losses due to anthracnose can be severe, as for Italy's Puglia olive oil sector, which incurred losses of $71 million due to the disease in 2011 (Butler, 2012). In Spain, overall industry income losses due to this disease were estimated to be $93 million per annum (Cacciola *et al*., 2012). In South Africa, anthracnose was reported on olives by Verwoerd (1929). Isolates from olives with anthracnose were later characterised morphologically as *Gleosporium fructigenum* f. sp. *chromogenum* by Gorter (1962). Baxter *et al*., (1983) identified isolates from anthracnose-affected olive fruit, based on cultural and morphological features, as *Colletotrichum acutatum*.

Anthracnose of olives can be caused by 14 *Colletotrichum* species, and these belong to three *Colletotrichum* complexes: *C. acutatum sensu lato* (*s.l.*), *C. boninense s.l*., and *C. gloeosporioides s.l.* (Moral *et al*., 2021). Inside the *C. acutatum s.l.* species complex *C. acutatum sensu stricto s.s*, *C. godetiae* and *C. nymphaeae* have been the most frequently reported, with *C. fiorinae*, *C. lupini*, *C. rhombiforme* and *C. simmondsii* reported less frequently (Moral *et al*., 2014; Materatski *et al*., 2018; Msairi *et al*., 2020). Species within the *C. gloeosporioides s.l.* species complex occur less than those of *C. acutatum s.l.* and include *C. aenigma*, *C. gloeosporioides* s.s., *C. kahawe* subp. *ciggaro*, *C. quenslandium, C. siamense* and *C. theobromicola* (Schena *et al*., 2014; Moral *et al*., 2014). Within the *C. boninense s.l.* species complex, only *C. karsti* has been associated with olive anthracnose in Italy (Schena *et al*., 2014). *Colletotrichum acutatum s.s.* has been reported in Australia, Brazil, Greece, Italy, Morocco, Portugal, South Africa, Tunisia and Uruguay (Gorter, 1956; Talhinhas *et al*., 2005; Sergeeva *et al*., 2008; Duarte *et al*., 2010; Chattaoui *et al*., 2016; Iliadi *et al*., 2018; Moreira *et al*., 2021; Msairi *et al*., 2020; Licciardello *et al*., 2022).

Damm *et al*., (2012) studied 331 isolates previously identified as *C. acutatum* or related species and identified 31 species using a sixgene phylogenetic approach. The 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers (ITS), partial sequences of the genes for glycelalderhyde-3-phosphate dehydrogenase (*gapdh*), chitin synthase 1 (*chs-1*), histone3 (*his3*), actin (*ACT*) and β-tubulin (*TUB2*) genes were used (Damm *et al*., 2012). With ITS, only 11 species could be identified (Damm *et al*., 2012). ITS and *TUB2* were enough to infer *Colletotrichum* species within the *C. acutatum* complex (Moral *et al*., 2021). No sequence analyses have been reported for *Colletotrichum* isolates from olive fruit in South Africa.

Olive anthracnose is known as a latent disease, as the symptoms are often only seen once fruit begins to ripen (Cacciola *et al*., 2012). Mature olive fruit is more susceptible to anthracnose due to changing pH and sugar content, as well as the changing structure and content of olive fruit cuticles (Da Silva, 2016). However, susceptible cultivars can show symptoms of disease on immature fruit drupes (Gomes *et al*., 2009). During unfavourable and non-fruiting seasons, *C. acutatum*

can be found on the surfaces of leaves and branches without appearance of any disease symptoms (Wharton and Diéguez-Uribeondo, 2004). Therefore, leaves and branches are where the pathogen overwinters, and are essential for inoculum survival (Crump, 2009). The latent nature of anthracnose and presence of overwintering structures make management of the disease difficult, as symptoms appear late in fruit maturity and can cause large yield losses.

Since unripe olives are more resistant to anthracnose than mature fruit, early harvesting to avoid the pathogen is effective and environmentally friendly (Moral *et al*., 2017). However, this method of disease management has disadvantages, since fully ripe fruit is required for natural black table olives, and immature fruit has low oil content (Moral *et al*., 2017). Therefore, use of resistant cultivars, if available, would be an effective control measure, and could be integrated with other control methods such as chemical and/or biological controls (Moral *et al*., 2017).

In South Africa, olive breeders select lines with anthracnose resistance to include in future breeding programmes. These selections usually occur in the field, and are dependent on a season that favours anthracnose development. Laboratory screening of olives from new olive tree lines for their resistance or tolerance towards anthracnose will aid breeders in future.

Chemical control of olive anthracnose in South Africa includes spraying of copper oxychloride, copper hydroxide, cuprous oxide and mancozeb (Croplife South Africa, 2024). While *Colletotrichum* spp. are sensitive to copper-containing fungicides, the pathogens are not effectively controlled under high disease pressure (Butler, 2012). To control anthracnose with copper fungicides, high numbers of spray treatments are required in rainy environmental conditions, as copper residues are easily washed away (Cacciola *et al*., 2012). This can create problems as copper ions in high concentrations can have adverse effects on soils and water near treated areas (Cacciola *et al*., 2012). Since the withdrawal of fungicides that include carcinogenic, mutagenic and reprotoxic (CMR) substances, according to the globally harmonised system of classification of chemicals, mancozeb is not be available for use on olives in South Africa from May 2025. In practice, copper fungicides are used on olives in South Africa to control anthracnose, including two sprays, one in spring and one in autumn. Therefore, other effective fungicides must be available for inclusion in efficient spray programmes.

The present study aimed to identify the *Colletotrichum* species associated with anthracnose of olives in South Africa and to improve current management of olive anthracnose, through identification of resistance in newly bred olive lines and possible effective fungicides. The objectives included: i) identifying the *Colletotrichum* species associated with symptomatic olives; ii) assessing the virulence of obtained *Colletotrichum* isolates on olive fruit; iii) assessing newly bred olive tree lines for anthracnose susceptibility and iv) determining the sensitivity of *Colletotrichum* isolates to four fungicide products that could be used for the control of olive anthracnose.

MATERIAL AND METHODS

Isolate collection and storage

Olive fruit with typical anthracnose symptoms and twigs with dieback were collected from olive orchards in the Western Cape region, South Africa, from April to August during 2014 to 2016. Collection locations are listed in Table 1 (Agter Paarl -33.589126 S, 18.860141 E; Paarl -33.709709 S, 19.031734 E; Durbanville -33.82950 S, 18.59325 E; Stellenbosch -33.916735 S, 18.919443 E). Lesioned fruit was placed in moist chambers, and incubated to allow conidiomata to sporulate. Shoots with dieback were surface sterilised (30 s in 70% ethanol, 2 min in 1 % NaOCl, 30 s in 70 % ethanol), and small sections of tissue between the dead and live parts of each shoot were removed and placed onto potato dextrose agar (PDA, Biolab) (39 g/L) amended with streptomycin (0.04 g/L). The PDA plates were incubated at 25°C, and inspected for *Colletotrichum* mycelial growth. From the incubated fruit, single conidium cultures were made from developed conidia plated onto PDA amended with streptomycin. Resulting pure cultures were stored in double-sterilised distilled water (dH_2O) in 14 mL capacity McCartney bottles, in the culture collection of the Department of Plant Pathology, Stellenbosch University (STEU).

Colletotrichum *species identification*

Molecular identification

Twenty *Colletotrichum* isolates were plated onto PDA Petri dishes and incubated at 25°C for approx. 2 weeks. DNA was extracted from mycelia of each isolate using a Wizard® Genomic DNA Purification Kit (Promega), with some amendments. Mycelia of each isolate were scraped into a 2 mL Eppendorf tube with 0.5 mg glass beads and 400 µL lysis buffer. The tubes were shaken for 5 min at 30 Hz in a Retsch Mixer Mill MM301 (Retsch). The Promega protocol was further followed, and DNA was eluted with 40 µL of nuclease-free water.

Table 1. Isolation details for *Colletotrichum acutatum* isolates identified in this study and reference isolates used in the phylogenetic analyses.

STEU isolate	Collector	Location	Cultivar	Date of collection	GenBank accession numbers		
					ITS	*TUB2*	*ACT*
8047	L. Mostert, A. Carlucci	Agter Paarl	Haas	May 2014	N/A	PV061016	PV061030
8048	L. Mostert, A. Carlucci	Agter Paarl	Picual	May 2014	PV035882	PV040775	PV040778
8049	L. Mostert, A. Carlucci	Agter Paarl	Picual	May 2014	PV035883	PV040776	PV040779
8050	L. Mostert, A. Carlucci	Agter Paarl	Picual	May 2014	PV035884	PV040777	PV040780
8061	C. Spies	Durbanville	Mission[a]	March 2015	PV055838	PV061017	PV061031
8064	C. Costa	Paarl	Mission	June 2016	PV055835	PV061010	PV061013
8065	C. Costa	Paarl	Mission	June 2016	PV055836	PV061011	PV061014
8067	C. Costa	Paarl	Mission	June 2016	PV055837	PV061012	PV061015
8068	C. Costa	Agter Paarl	Vdl sl A, tree seedling	June 2016	PV055839	PV061018	PV061032
8069	C. Costa	Agter Paarl	Vdl sl A, tree seedling	June 2016	PV055840	PV061019	PV061033
8070	C. Costa	Agter Paarl	Vdl sl A, tree seedling	June 2016	PV055841	PV061020	PV061034
8071	C. Costa	Agter Paarl	Vdl sl A, tree seedling	June 2016	PV055842	PV061021	PV061035
8072	C. Costa	Agter Paarl	Vdl sl A, tree seedling	June 2016	PV055843	PV061022	PV061036
8220	J. Scrimegeour	Paarl	Mission	August 2016	PV055844	PV061023	PV061037
8221	J. Scrimegeour	Paarl	Mission	August 2016	PV055845	PV061024	PV061038
8222	J. Scrimegeour	Paarl	Mission	August 2016	PV055846	PV061025	PV061039
8223	J. Scrimegeour	Paarl	Mission	August 2016	PV055847	PV061026	PV061040
8226	J. Scrimegeour	Paarl	Mission	August 2016	PV055848	PV061027	PV061041
8227	J. Scrimegeour	Paarl	Mission	August 2016	PV055849	PV061028	PV061042
8229	F. Halleen	Stellenbosch	Unknown	August 2016	PV055850	PV061029	PV061043

[a] Isolate was obtained from twig dieback symptom. All other isolates were from symptomatic olive fruit.

For the identification of the *Colletotrichum* isolates, three gene regions were amplified and sequenced. The internal transcribed spacer (ITS) gene region was amplified with the ITS4 and ITS5 primer pair (White *et al*., 1990), the partial beta-tubulin gene region (*TUB2*) using T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) primer pair and the partial actin (*ACT*) gene using the ACT-512F and ACT-783R primer pair (Carbone and Kohn 1999). Each 20 µL reaction comprised 2 µL of DNA, 10 µL Amplicon Taq DNA Polymerase Master Mix RED (ThermoFisher), 7 µL of ddH_2O and 0.50 µL (10 mM) of each primer.

The PCR conditions for ITS consisted of an initial denaturation of 5 min at 94°C, followed by 35 cycles each of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C and a final extension of 72°C for 6 min. The reaction conditions for *TUB2* consisted of initial denaturation at 94°C for 5 min, followed by 36 cycles each of 94°C for 45 s, 58°C for 30 s for the annealing, 72°C for 90 s and a final extension of 72°C for 6 min. For the *ACT* gene region, reaction conditions consisted of initial denaturation of 94°C for 5 min, followed by 40 cycles each of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 7 min.

The PCR products were separated and visualised with electrophoresis on a 1% (w/v) agarose gel which was stained with SYBR Safe DNA gel stain (Thermo Fisher Scientific) in TAE running buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.5). PCR products were sequenced in both directions at the Central Analytical Facilities (CAF), Stellenbosch University, South Africa.

Consensus sequences were generated from the forward and reverse sequences in Geneious 11.1.5 (Biomatters Ltd.). To determine the *Colletotrichum* species complex, preliminary identification was based on Basic Local Alignment Search Tool for nucleotides (BLASTn) searches against the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) nucleotide database. Reference sequences of the *C. acutatum* species complex (Table S1), based on Damm *et al*., (2012) and Vieira *et al*., (2020), were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank). Consensus sequences from each gene region were aligned with reference sequences using MAFFT v.1.3.6 with the L-INS-I method (Katoh and Standley, 2013) in Geneious. Concatenated sequences were generated. A maximum likelihood (ML) phylogeny was constructed for the 20 *Colletotrichum* isolates using the ITS, *TUB2* and *ACT* gene regions using PhyML v2.2.3 (Guindon *et al*., 2010) under the general time reversible (GTR) model. The gamma distribution and proportion of non-variable sites

were estimated. Bootstrap support values were calculated with 1000 replicates and clades with bootstrap support of 70% or more were considered significant and supported (Hills and Bull, 1993).

Morphological and cultural characterisation of isolates

Eight *Colletotrichum* isolates were plated onto water agar containing sterile dried carnation leaves and were incubated at 25°C in the dark until sporulation. Microscope preparations of the conidial masses were made in 70% lactic acid and observed at 1000× magnification using a Nikon Eclipse E600 compound microscope with a Nikon DS-Ri2 digital camera attachment. Lengths and widths of 30 conidia per isolate were measured. The eight *Colletotrichum* isolates were plated onto PDA and incubated at 25°C for 14 d in the dark and resulting colony colours were determined using Rayner's colour chart (Rayner, 1970).

Virulence assessment of isolates

The virulence of twenty isolates was assessed on cultivars Manzanilla (anthracnose susceptible) and Mission (moderately susceptible). The isolates were each plated onto water agar (WA) (12 g/L) containing sterile carnation leaves, to induce conidium formation, and were incubated at 25°C for 5 days in the dark. A conidium suspension of each isolate was prepared by vigorously mixing a carnation leaf covered with acervuli in 2 mL of sterile water using a vortex mixer. The suspension was then filtered using sterile cheesecloth and conidium concentration was adjusted to 1×10^6 conidia mL^{-1} using sterile water. Olive fruit was collected in March 2018. The fruits were surface sterilised for 30 s in 70% ethanol, 30 s in 1% NaOCl and 30 s in 70% ethanol, and then allowed to dry in a laminar flow cabinet. One wound per fruit was made using a sterile needle, after which each fruit was inoculated by pipetting 10 mL of conidium suspension directly onto the wound. Six fruits were inoculated with each isolate treatment, and the trial was repeated once. Plastic containers were sprayed with 70% ethanol and left to dry in a laminar flow cabinet. Sterile tissue paper was placed in each container and moistened with 50 mL of sterile water. In each container, three fruits per treatment and three treatments were incubated (nine fruits per container), in a randomised arrangement. Twelve control fruit were inoculated with sterile water. The containers were then incubated at 25°C under a 12 h light 12 h dark regime for 8 d, and the the fruits were assessed for lesions every 24 h. The numbers of fruit infected at each assessment interval was recorded and lesions size was measured after 8 d. Analyses of Variance (ANOVA) were used to compare each cultivar for experiments and isolates, using the GLM procedure and using the LSD t-test (at $P = 0.05$). The three most virulent isolates were selected based on their cultivar reactions and consistency among fruit replicates.

Susceptibility of different olive lines

Olive fruit of 18 newly bred lines and six cultivars with known anthracnose resistance (Moral *et al*., 2017) were provided by Carlo Costa in March and April 2019 (ARC Infruitec-Nietvoorbij), from unsprayed trees. The cultivars were: Haas (highly susceptible to anthracnose); Kalamata (susceptible); Mission (moderately susceptible); Nandi (resistant); Coratina (resistant) and Frantoio (highly resistant). Three trials were carried out where black table olive, green table, and oil olive lines were assessed.

Fruit was collected at three time during 2019 from which three trials were carried out. For the first trial, fruit were collected on 12 March 2019, and included the newly bred olive lines: B03.30 (3/4 ripe); B07.12 (1/2 ripe); B07.26 (1/2 ripe); B12.12 (1/2 ripe); and B5.22 (1/2 ripe) which were tested with the reference cultivars: Coratina (green); Frantoio (3/4 ripe); Haas (green); Haas (1/2 ripe); Kalamata (ripe); Mission (green) and Mission (1/2 ripe). For the second trial, fruit were collected on 27 March 2019, and included the newly bred lines: A29.32 (green); B12.42 (1/4 ripe); B43.16 (1/4 ripe); C31.26 (green); C36.02 (1/4 ripe); and VC14.40 (1/4 ripe) which were tested with the reference cultivars: Coratina (green); Frantoio (green); Haas (green); Nandi (green); Kalamata (ripe) and Mission (green). For the third trial, fruit were collected on 16 April 2019, and included the newly bred lines: A15.36 (green); A16.42 (green); A23. CD (green); B04.22 (1/4 ripe); B11.34 (1/4 ripe); B22.02 (ripe); and B34.40 (ripe). The fruit of these lines were tested with fruit from the following reference cultivars: Coratina (green); Frantoio (green); Kalamata (ripe); Mission (green); and Mission (ripe).

The three most virulent isolates (STEU 8049, STEU 8220, and STEU 8226) were each cultured on WA with sterile carnation leaves for 5 d. A conidium suspension was prepared from each isolate, as described (above), and was adjusted to 1×10^6 conidia mL^{-1}. Fruit (unwounded) were surface sterilised as described (above), were left to air dry in laminar flow cabinet, and were then each submerged in conidium suspension for 1 min. For the negative controls, ten fruit per cultivar were treated with sterile water instead of the conidium suspension. The fruit were then incubated at 25°C with a

12-h photo period in sterile moisture chamber (ten fruit per chamber). Each treatment was inoculated onto ten olive fruits, and was repeated three times.

The fruits were visually assessed for lesions every 24 h from 5 d to 8 d post-inoculation. To determine resistance levels of the fruit, the number of infected fruits at each assessment interval was recorded using a visual disease severity index for lesions on fruits (Moral *et al*., 2008). If no lesion was present on an olive, fruit severitywas recorded as 0, and lesions covering the entire fruit were recorded as 5. First sign of lesion development was scored as 1, lesions covering a quarter of the fruit as 2, covering half of the fruit as 3, and covering three quarters of the fruit were recorded 4.

Mean disease severity indices for each cultivar and line tested were used to calculate the McKinney's Index (MKI; McKinney, 1923). In the McKinney's Index the disease severity per cultivar was converted into a percentage of the maximum severity level, using the following equation: McKinney's Index = $[S(n_i\text{-}i) / 5 \times N] \times 100$, where i represents the infection severity (0-5), n_i is the number of fruit with severity and N is the total number of fruit for that replicate.

ANOVA was carried out to compare trials and olive cultivars, using the GLM procedure, and means were compared using LSD t test (at $P = 0.05$).

Fungicide sensitivity of Colletotrichum *isolates*

Fungicide inhibition of mycelium growth of the 20 *Colletotrichum* isolates was determined for the fungicides boscalid + pyraclostrobin (BASF), dodine (Campbell Chemicals), cyprodinil + fludioxinil (Syngenta) and thiram (Villa Crop). The concentration range for each fungicide is indicated in Table S2. For mycelium growth inhibition, each concentration was added to molten PDA at 55°C. PDA containing no fungicide was used as negative controls. Two replicates per trial were conducted, and the trials were repeated. Petri plates containing the media were incubated at 25°C for 8 d in the dark. Colony diameters were measured, and growth of each isolate at different fungicide concentrations was determined by calculating the mean diameters of the two colonies of each isolate.

Conidium germination of five *Colletotrichum* isolates was assessed as affected by the four fungicides, using an adjusted method of Gramaje *et al*., (2009). A conidium suspension was prepared by placing a carnation leaf with sporulating *Colletorichum* acervuli in 5 mL of half-strength potato dextrose broth (12 g/L) (Biolab). The conidium suspension concentration was determined with a haemocytometer, and was adjusted to 1×10^5 conidia mL^{-1}. One millilitre of conidium suspension each was dispersed into 2 mL Eppendorf tubes, and 1 mL of fungicide was added to each tube to obtain the correct fungicide concentration. Six concentrations per fungicide were tested (Table 2), and the negative control contained only broth. A total volume of 36 µL (containing 3600 conidia) was pipetted per microscope slide, a coverslip was placed on, and the slides were incubated in a moist chamber at 25°C in the dark. Conidium germination was assessed after 24 h by evaluating the germination of 100 conidia per slide. Conidia were considered germinated when the germ tubes had exceeded one-half of the length of the conidia. Two repeats of each isolate and fungicide concentration, were made and the trial was repeated once.

The percentage conidium inhibition per isolate per fungicide concentration was calculated as a proportion of the experimental controls. Percentage inhibition was fitted against fungicide concentration using the Mitsherlich function for boscalid + pyraclostrobin and thiram [PInhibition = 100(1-exp(-rate × concentration] and Gompertz for cyprodinil + fludioxonil and dodine [PInhibition = a*exp(-exp(-b*(LConc-c)))] (Koya and Goshu, 2013). Concentrations at which 50% of the mycelium growth or conidium germination was inhibited (EC_{50}) were calculated using Mitsherlich parameters [EC_{50} = log(1-50/100)/(-rate)] or Gompertz [LEC_{50}=((log(-log(50/a)))/-b)+c]. Regression parameters obtained were subjected to ANOVA for each fungicide, where trials were considered as block replicates for isolates. Standard residuals were tested using the Shapiro-Wilk test (Shapiro and Wilk, 1965), to determine deviations from normality. Isolate means were compared by calculating Fisher's least significance at $P = 0.05$ (Ott, 1998), and that probability level was considered significant for all tests.

RESULTS

In the field, mostly anthracnose affected olive fruits and occasionally twig dieback were observed (Figure 1). Twenty *Colletotrichum* isolates were obtained, of which 19 were from diseased fruit, and one was from twig dieback (Table 1).

Colletotrichum *species identification*

All 20 *Colletotrichum* isolates formed a monophyletic clade with the *C. acutatum s.s.* type isolate (CBS 112996), with 100% bootstrap support (Figure 2). Isolate growth in culture was typical of *C. acutatum*, with buff to salmon pink mycelium. For one isolate (STEU 8061), the colonies were pale grey with white aerial mycelium (Figure 3 a and b). Acervuli contained orange masses of conidia

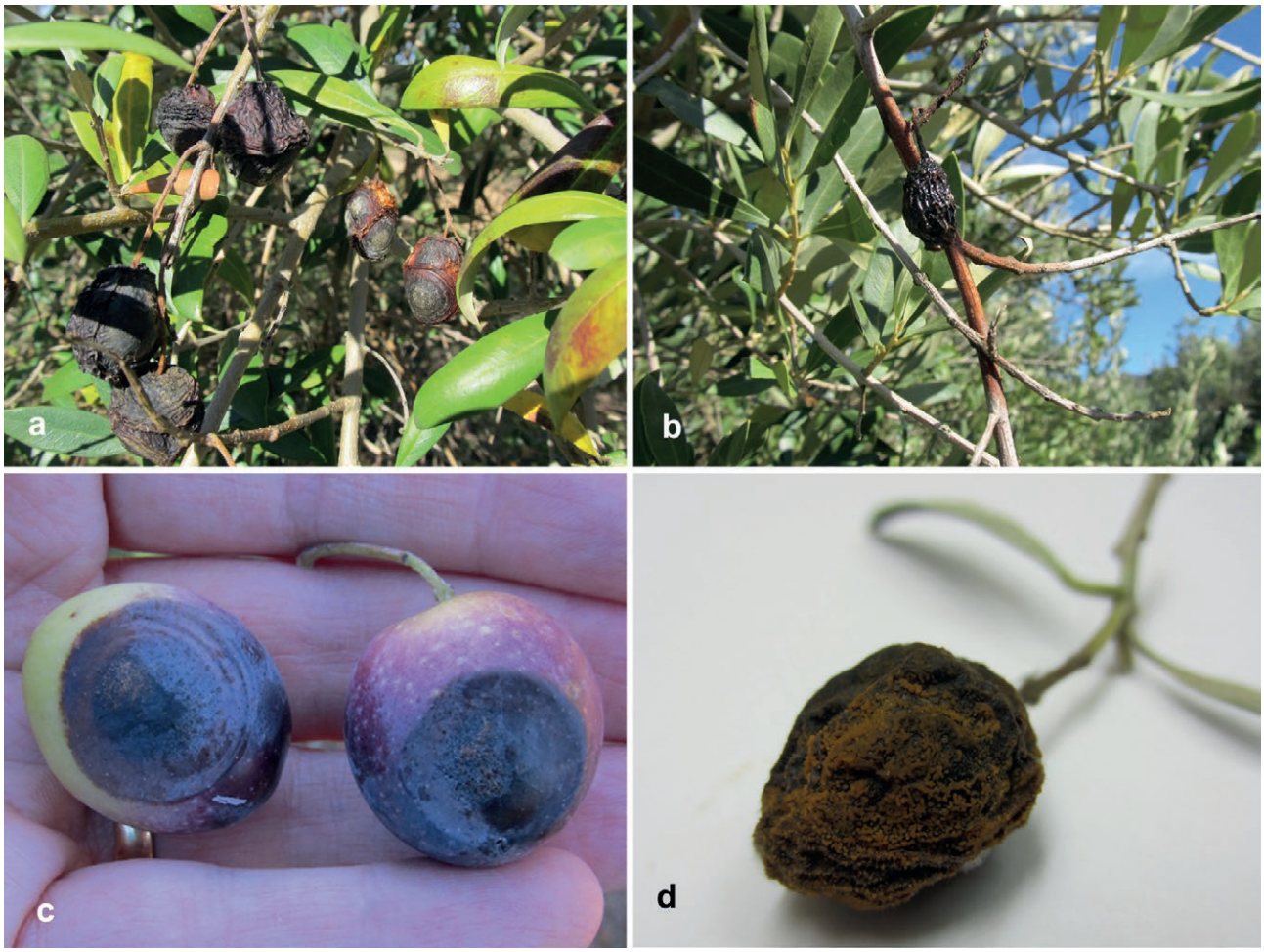

Figure 1. Symptoms associated with *Colletotrichum acutatum* infections. Fruit in an orchard with anthracnose symptoms (a) and infected olive drupe with twig showing dieback (b). Typical olive fruit symptoms with concentric rings (c) and orange masses associated with acervuli present on infected fruit (d).

on the carnation leaves (Figure 3 c and d). Conidia were hyaline, aseptate and fusiform, and measured (12-)14-16(-18) × 4-5 mm, averaged for the eight isolates (Figure 3 e and f) (Table S3).

Virulence assessments of isolates

Mean lesion lengths varied from 3.4 to 12.9 mm on Mission and 3.4 to 12.8 mm on Manzanilla (Figure 4). Isolates STEU 8049, STEU 8220 and STEU 8226 with respective average lesion lengths of 12.8, 10.1 and 11.2 mm on Manzanilla and 12.9, 11.1 and 11.3 mm on Mission, were identified as the most virulent *C. acutatum* isolates (Table S2). Two isolates (STEU 8048 and STEU 8223) produced small lesions on both cultivars, which were not significantly different from those from the water experimental control (Table S4). Isolate STEU 8061, which was from a dieback twig, was pathogenic on both fruit types and formed lesions of mean length 8.8 mm on Manzanilla and 9.8 mm on fruit of Mission.

Susceptibility of newly bred olive lines

Mean infection severity for the repeat of each trial was not significantly different ($P = 0.1921$ for trial 1, 0.6298 for trial 2, and 0.4600 for trial 3, so results of the experimental repeats were combined. There were significant cultivar and time after inoculation interactions ($P < 0.0001$), so the results are provided in this format in Table 2. For the reference cultivars, Kalamata (ripe) was the most susceptible

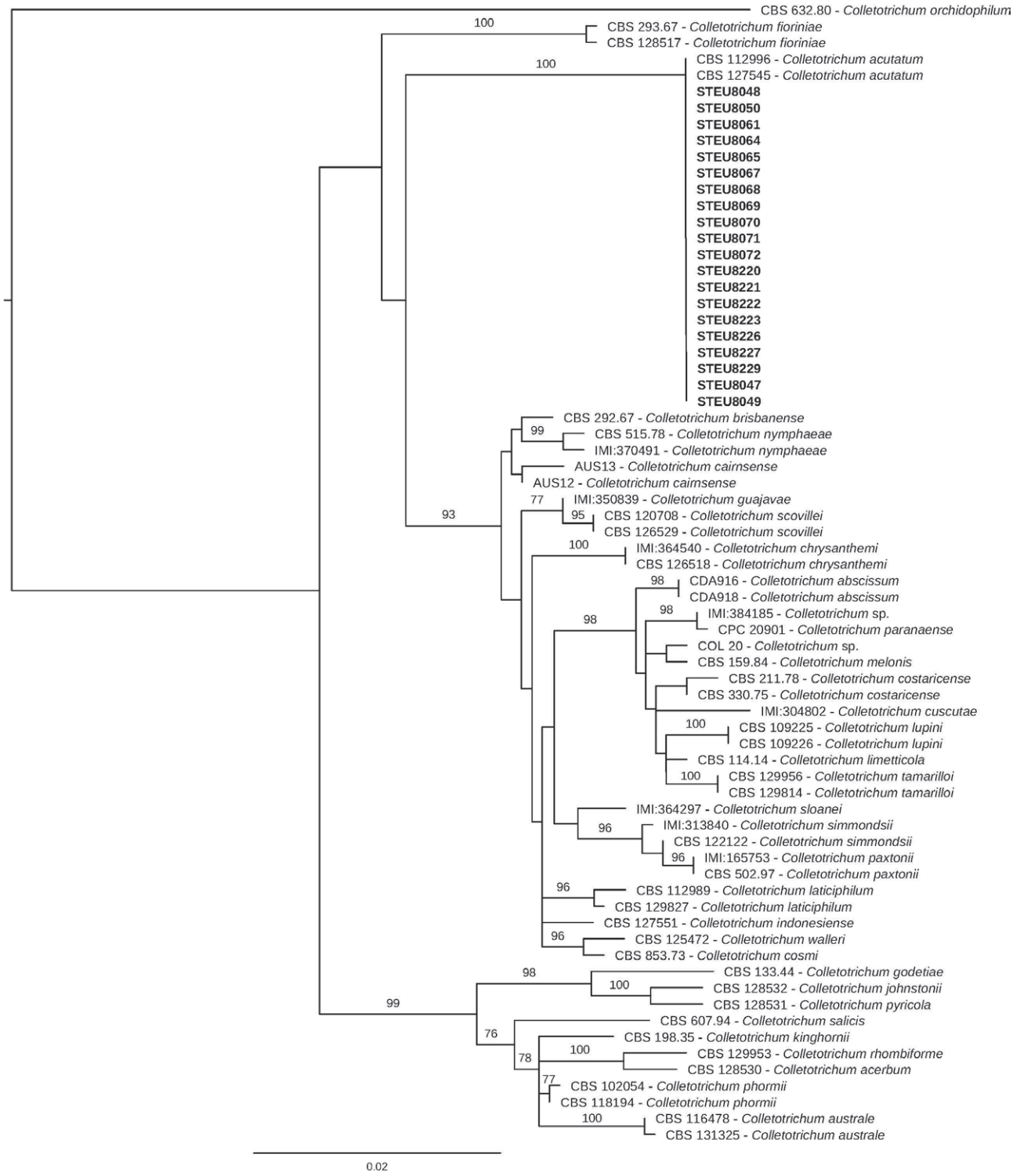

Figure 2. Maximum likelihood (ML) phylogenetic tree for *Colletotrichum acutatum* species complex based on *ACT*, *TUB2* and *ITS* gene regions. Bootstrap values of 70% and greater are shown. *Colletotrichum orchidophilum* (CBS 128530) was used as the outgroup and sequences generated in the present study are in bold font.

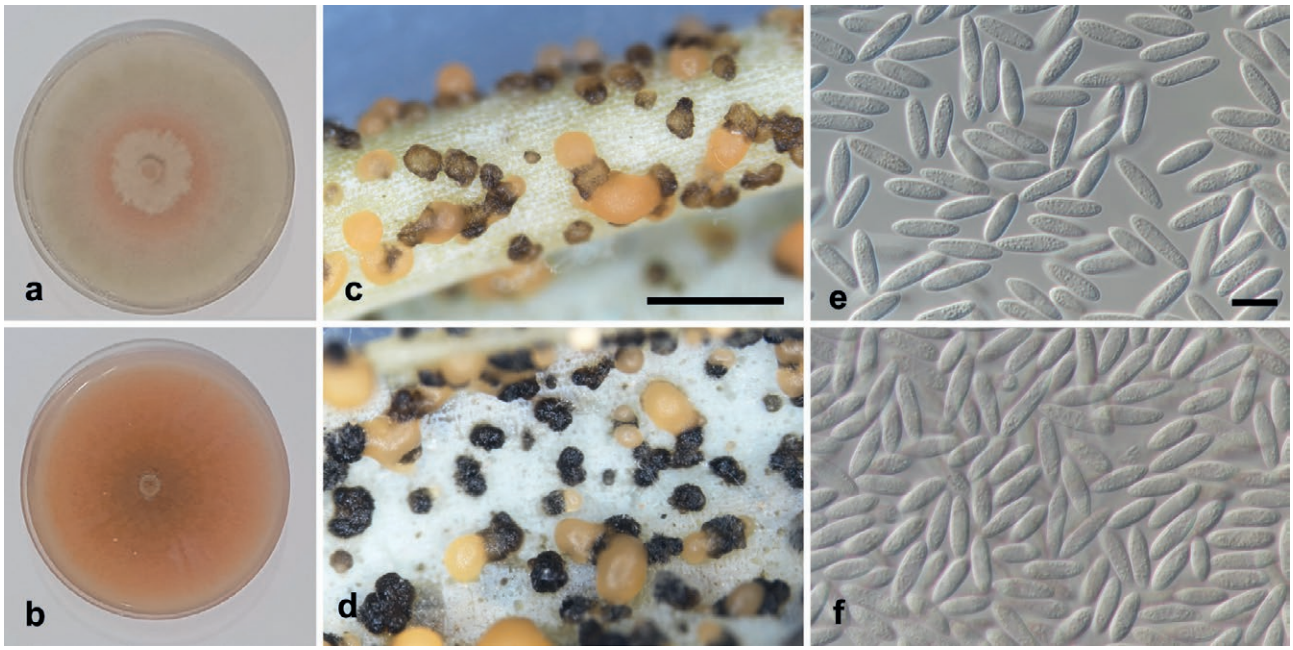

Figure 3. Cultural growth of *Colletotrichum acutatum* on PDA after two weeks (a and b), acervuli formed on carnation leaves (c and d) and conidia (e and f). Scale bar in c = 1000 μm (applies to d); e = 10 μm (applies to f). STEU8061 is shown in a, c and e; STEU8229 is shown in b, d and f.

Figure 4. Olive fruits after inoculations with different STEU isolates of *Colletotrichum acutatum*, assessed for virulence on olive fruits of Manzanilla and Mission. Control fruit (left and right ends of each row) were inoculated with sterile water as inoculation controls.

(in all three trials). Four potential oil olive lines showed resistance of which A29.32 (green) and C31.26 (green) were identified as highly resistant, and A16.42 (green) and C36.02 (1/4 ripe) were identified as resistant. Of the green table olive lines, B11.34 (1/4 ripe) and B12.42 (1/4 ripe) as highly resistant, B04.22 (1/4 ripe) as resistant, and B07.26 (1/2 ripe) was identified as moderately susceptible. Only one potential black table olive B34.40 (ripe) was identified as moderately susceptible (Table 2).

Fungicide sensitivity of Colletotrichum *isolates*

For the mycelium growth inhibition experiments, there were no significant differences between experiments [boscalid + pyraclostrobin ($P = 0.232$), dodine ($P = 0.924$), cyprodinil + fludioxonil ($P = 0.690$), thiram ($P = 0.593$)], so the results for the three experiments were combined. Sensitivity of the isolates was significantly different ($P < 0.0001$) for each fungicide, and-

Table 2. Mean infection severity calculated using the McKinney's Index, for the most virulent isolates of *Colletotrichum acutatum* tested on 18 newly bred olive lines and six olive reference cultivars.

Cultivar	Ripeness	Olive type of new lines	Mean infection severity[a]			
			At 120 h	At 144 h	At 168 h	At 192 h
Trial 1 (fruit collected 12 March 2019)						
Kalamata	ripe		74.67h[b]	99.33a	100.00a	100.00a
Haas	green		58.33j	89.00ed	97.67ab	100.00a
Haas	1/2 ripe		81.00g	98.67a	100.00a	100.00a
B12.12	1/2 ripe	oil	34.00mn	67.00i	83.67fg	96.33ab
Mission	1/2 ripe		29.00o	57.67j	82.33fg	94.00bc
B5.22	1/2 ripe	oil	28.67o	57.67j	85.67ef	91.67cd
Mission	green		21.33p	40.00l	57.67j	83.33fg
B03.30	3/4 ripe	oil	0.00u	2.33tu	30.33on	47.67k
B07.12	1/2 ripe	oil	1.67tu	12.00rq	22.42p	38.33ml
B07.26	1/2 ripe	oil	0.33u	3.67stu	14.33q	28.67o
Frantoio	3/4 ripe		1.00tu	2.33tu	5.00st	7.67rs
Coratina	green		0.00u	0.00u	0.00u	0.00u
Trial 2 (fruit collected 27 March 2019)						
Kalamata	ripe		59.33e	73.33c	87.00b	98.67a
B43.16	1/4 ripe	green table	18.00j	32.67h	53.67f	65.00d
Haas	green		11.00k	24.00i	41.67g	64.00d
Mission	green		11.67k	20.00j	32.33h	60.33e
VC14.40	1/4 ripe	green table	0.67on	1.67on	7.00l	17.67j
C36.02	1/4 ripe	green table	0.67on	3.33mn	7.33l	13.67k
Nandi	green		0.33on	0.67on	2.00on	5.33ml
B12.42	1/4 ripe	oil	0.00o	0.00o	0.33on	3.00mno
A29.32	green	green table	1.67on	1.67on	1.67on	2.00on
C31.26	green	green table	0.00o	0.00o	0.00o	0.33on
Coratina	green		0.00o	0.00o	0.00o	0.00o
Frantoio	green		0.00o	0.00o	0.00o	0.00o
Trial 3 (fruit collected 16 April 2019)						
Kalamata	ripe		34.33j	66.67e	82.00c	100.00a
Mission	ripe		14.33op	56.33g	72.00d	90.33b
B22.02	ripe	green table	18.00n	44.00i	63.33f	79.67c
A15.36	green	green table	19.67mn	29.33k	41.67i	51.67h
Mission	green		1.33uv	11.00q	16.67no	34.33j
A23.CD	green	oil	4.33surt	11.00q	14.67op	23.00l
B34.40	ripe	black table	2.67suvt	4.67srt	12.33qp	22.00ml
B04.22	1/4 ripe	oil	0.00v	2.67suvt	6.00r	11.00q
A16.42	green	green table	1.00v	1.67uvt	1.67uvt	5.67sr
B11.34	1/4 ripe	green table	0.00v	0.00v	0.00v	0.00v
Coratina	green		0.00v	0.00v	0.00v	0.00v
Frantoio	green		0.00v	0.00v	0.00v	0.00v

[a] Olive fruit was dip inoculated with *C. acutatum* conidium suspensions and incubated for 8 d. Lesion development was recorded from day 5 to day 8. For each olive line ten olive fruit were inoculated and this was repeated twice, the mean severity index was calculated from 30 observations.

[b] Means accompanied by the same letter in each column and row do not differ significantly ($P < 0.0001$) for separate trials.

mean EC_{50} values for the isolates are presented in Table 3. Ranges in mean EC_{50} values were: boscalid + pyraclostrobin, 0.20 to 0.51 µg mL^{-1}; dodine, 93.36 to 897.21 µg mL^{-1}, cyprodinil + fludioxonil, 0.03 to 0.06 and thiram, 19.32 to 104.20 µg mL^{-1} (Table 3). The isolates of *C. acutatum* were least sensitive to dodine and thiram,

Table 3. Mean colony diameter growth inhibition EC_{50} values (μg mL^{-1}) determined for 20 *Colletotrichum acutatum* isolates exposed to four different commercial fungicides.

STEU isolate	Boscalid + pyraclostrobin	Dodine	Cyprodinil + fludioxinil	Thiram
8047	0.46	499.49	0.03	60.72
8048	0.33	502.36	0.04	97.47
8049	0.40	277.62	0.04	54.82
8050	0.45	657.01	0.03	41.30
8061	0.30	473.19	0.04	75.81
8064	0.33	587.83	0.04	27.09
8065	0.41	365.33	0.04	37.08
8067	0.36	652.65	0.04	87.53
8068	0.30	820.82	0.04	75.26
8069	0.30	799.19	0.04	52.60
8070	0.35	755.78	0.04	104.20
8071	0.20	770.43	0.06	98.81
8072	0.26	897.21	0.03	42.18
8220	0.37	359.68	0.03	53.49
8221	0.50	800.09	0.03	41.51
8222	0.51	741.57	0.03	79.52
8223	0.49	635.75	0.04	57.67
8226	0.42	561.27	0.04	29.14
8227	0.38	93.36	0.04	69.35
8229	0.36	841.20	0.04	19.32

[a] Colony diameters for each isolate were measured twice (perpendicularly and horizontally) at different concentrations of fungicides, and were each determined by calculating the mean diameter of two colonies.

Table 4. Mean EC_{50} values (μg mL^{-1}) calculated from conidium germination inhibition determined for five *Colletotrichum acutatum* isolates.

STEU isolate	Boscalid + pyraclostrobin	Dodine	Cyprodinil + fludioxinil	Thiram
8047	1.53	6.67	0.16	0.04
8049	0.02	10.50	0.55	0.03
8220	0.89	8.00	0.41	0.06
8221	1.48	6.11	0.30	0.09
8226	1.02	5.07	0.37	0.07

[a] One-hundred conidia were counted for two replicates per fungicide concentration per isolate and the trial was repeated twice.

but were most sensitive to boscalid + pyraclostrobin and cyprodinil + fludioxinil.

For conidium germination, mean proportions for the experiments were not significantly different from each other [boscalid + pyraclostrobin, $P = 0.497$; dodine, $P = 0.310$; cyprodinil + fludioxonil, $P = 0.697$ for dodine, $P = 0.517$ for thiram], so results from the experiments were combined. There were significant isolate differences for all the fungicides ($P < 0.03$) (Table 4). The conidium germination results showed that *C. acutatum* was least sensitive to dodine (EC_{50} range 5.05 to 10.50 μg mL^{-1}). The EC_{50} ranges from the other fungicides treatments were 0.02 to 1.53 μg mL^{-1} for boscalid + pyraclostrobin, 0.16 to 0.55 μg mL^{-1} for cyprodinil + fludioxinil and 0.03 to 0.09 μg mL^{-1} for thiram.

DISCUSSION

Only *C. acutatum s.s.* was identified as the cause of olive anthracnose plant samples collected from in the Western Cape of South Africa. In Tunisia and Uruguay, *C. acutatum s.s.* was also the major species associated with olive anthracnose (Chattaoui *et al.*, 2016; Moreira *et al.*, 2021), and in Pakistan, only *C. acutatum* has been associated with olive anthracnose (Nawaz *et al.*, 2023). However, other major olive-producing countries have more than one *Colletotrichum* species associated with olive anthracnose. In Portugal, the major species is *C. nymphaeae* and to a lesser extent *C. godetiae*, in Spain, it is *C. godetiae* followed by *C. nymphaeae*, *C. acutatum* and *C. fructicola* and in Italy, the three major species are *C. godetiae* followed by *C. nymphaeae* and *C. acutatum* (Moral *et al.*, 2021). In Australia, eight species of *Colletotrichum* were identified from olives (Moral *et al.*, 2021).

In the present study, a polyphasic approach for species identification was followed, which included colony characters and conidium dimensions. The phylogenetic analyses that included three gene regions, ITS, *ACT* and *TUB2* determined all the isolates were *C. acutatum s.s.* Of the two most diagnostic genes for *C. acutatum* s.l., *TUB2* was slightly better than *gapdh* for resolving species (Damm *et al.*, 2012). Even though multiple gene regions have been employed to distinguish *Colletotrichum* species, it has been shown that ITS combined with *TUB2* is generally sufficient for species identification within the *C. acutatum* s.l. complex (Moral *et al.*, 2021).

In the present study, unwounded detached fruit from 18 newly bred olive lines, as well as five reference cultivars, were used to evaluate cultivar susceptibility to *C. acutatum* infections. Four oil olive lines and three green table lines were identified as resistant to this fungus. Severity of infection differed between the cultivars, and and cultivar differences have also been noted in previous studies (Moral *et al.*, 2008; Moral and Trapero, 2009; Talhinhas *et al.*, 2011; Cacciola *et al.*, 2012; Moral *et al.*, 2014). Moral *et al.*, (2017) evaluated 308 cultivars for anthracnose susceptibility, and categorised the cultivars as highly resistant, resistant, moderately susceptible,

susceptible, or highly susceptible. Of these, 32 cultivars were highly resistant, and 61 were resistant to *C. acutatum* infections.

Different cultivar reactions to *C. acutatum* infection could be a result of several genes interacting between the olive host and this pathogen (Wharton and Dieguez-Uribeondo, 2004). This type of resistance is known as dynamic incompatibility, where pathogen avirulence gene products cause host resistance genes to inhibit pathogen's growth under specific physiological conditions, such as fruit maturity (Wharton and Dieguez-Uribeondo, 2004). The different reactions of cultivars to *C. acutatum* infections could also be due to varying cuticle thicknesses and chemical compounds on fruit surfaces of different cultivars (Gomes *et al*., 2012). These characteristics have been shown to differ among the different cultivars (Gomes *et al*., 2012; Da Silva, 2016). Cuticles of olive fruit have a large amounts of lipids, which act as barriers for conidia adhesion, which slows *C. acutatum* infection processes (Da Silva, 2016).

Physical disease management strategies which are being used to control anthracnose of olives includes early harvesting of very susceptible cultivars to prevent secondary infections occurring, pruning trees to improve air circulation and reduce humidity because high levels are favourable for the pathogen, and removal of infected twigs and mummified fruit to reduce the amounts of inoculum (Cacciola *et al*., 2012). In the present study, one *C. acutatum* isolate, obtained from a twig with dieback, also induced symptoms on Manzanilla and Mission. This illustrates the importance of the removal of infected twigs from orchards to reduce pathogen inoculum.

The four fungicides tested in the present study inhibited the mycelium growth and conidium germination of *C. acutatum* isolates. For mycelium growth inhibition, boscalid + pyraclostrobin and cyprodinil + fludioxinil gave lower EC_{50} values than thiram or dodine. However, thiram gave lowest EC_{50} values for conidium germination inhibition, followed by cyprodinil + fludioxonil and boscalid + pyraclostrobin. Boscalid + pyraclostrobin is registered in South Africa for the control of *Colletotrichum* spp. on chillies, peppers, strawberries and tree nuts. The active ingredient pyraclostrobin has been shown to completely inhibit growth of *C. gloeosporioides* and *C. truncatum* which causes anthracnose on avocadoes, mangoes, papayas and peppers (Rampersad and Teelucksingh, 2012). Pyraclostrobin is a quinone outside inhibitor fungicide, and boscalid is a succinate dehydrogenase inhibitor (SDHI), with both fungicides inhibiting mitochondrial respiration in fungi (FRAC, 2025). In the present study, no mycelium growth was observed for nine of the isolates at the greatest tested concentration of boscalid + pyraclostrobin. Pyraclostrobin strongly inhibits conidium germination, whereas boscalid inhibits mycelium growth more (Piccirillo *et al*., 2018; Yang *et al*., 2021; Usman *et al*., 2022). EC_{50} values for four of the five *C. acutatum* isolates' (1.02 to 1.53 mg/mL) for conidial germination were greater than those for mycelium growth inhibition (EC_{50} = 0.20 to 0.51 mg mL^{-1}). Everett and Timudo-Torrevilla (2007) also assessed boscalid + pyraclostrobin *in vitro* on PDA for effects on *C. acutatum* isolates from avocados, and reported greater EC_{50} values for conidium germination (7 mg mL^{-1}) than mycelium growth (0.2 mg mL^{-1}).

Dodine (in the guanidine fungicide group) is used to control diseases such as leaf spot, brown rot, blossom rot, Fusarium wilt, and downy mildew on different tree fruit crops (Pesticide Property Database, 2019). The mode of action of dodine is unknown, but is proposed to be cell membrane disruption in some pathogens, and has been shown to inhibit conidium germination in *Venturia oleagineum,* which causes olive leaf spot (Rongai *et al*., 2012; Almadi *et al*., 2024). This fungicide was the least effective of those assessed in the present study, giving the greatest EC_{50} values for inhibition of *C. acutatum* mycelium growth and conidium germination. Dodine was tested and found ineffective for inhibiting mycelium growth of *C. acutatum* from Uruguay, and is not recommended for anthracnose control on olives (Moreira *et al*., 2024).

Cyprodinil + fludioxonil is a translaminar systemic and contact fungicide, including anilinopyrimidine (cyprodinil) and phenylpyrrole (fludioxinil) active ingredients. Cyprodinil inhibits amino acid biosynthesis in fungi, and fludioxonil is a contact fungicide that inhibits conidium germination, and the tube and mycelium growth. This fungicide mixture has been registered in South Africa for the control of *Colletotrichum* spp. of berries, strawberries, and tropical fruit including avocado. The two fungicide mixtures were tested in the present study, rather than the single actives, to aid decision-making for product registration trials, and to ultimately provide options for disease management for olive growers. Both of these mixtures have been registered for *Colletotrichum*/anthracnose on other crops in South Africa.

Thiram is a contact fungicide, and is registered in South Africa for the control of scab of apples and pears, blossom blight of stone fruit trees, leaf curl of peaches, and for apple seed decay and seedling rot. Three thiram products have been assessed for effects on *C. acutatum* isolates from strawberries, and were found to be moderately effective for inhibition of mycelium growth, but very effective for inhibiting conidium germination (Es-Soufi *et al*., 2018).

In conclusion, *C. acutatum s.s.* is the only species to be associated with olive anthracnose in South Africa. Seven newly bred olive lines were identified as resistant to *C. acutatum* infection. These potential olive lines can be used by breeders to produce commercial olive cultivars that have increased tolerance to this pathogen. Potential fungicides for the chemical control of olive anthracnose were tested for their ability to inhibit mycelial growth and conidial germination. Boscalid + pyraclostrobin, cyprodinil + fludioxonil, and thiram have potential, and require further assessments in olive orchards. Effects of application of the fungicides on olive fruit and oil residues have yet to be determined.

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