Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers

Characterization of *Colletotrichum* strains associated with olive anthracnose in Sicily

Grazia LICCIARDELLO[1,*], Juan MORAL[2,3], Maria Concetta STRANO[1], Paola CARUSO[1], Marika SCIARA[1], Patrizia BELLA[4], Guido SORRENTINO[1], Silvia DI SILVESTRO[1]

[1] *Consiglio per la Ricerca in agricoltura e l'analisi dell'Economia Agraria-Centro di ricerca Olivicoltura, Frutticoltura e Agrumicoltura (CREA), Corso Savoia, 190 Acireale (CT), Italy*
[2] *Department of Agronomy, Maria de Maeztu Excellence Unit, University of Córdoba. Edif. C4, Campus de Rabanales, 14071 Córdoba, Spain*
[3] *Department of Biology, College of Science and Mathematics, California State University, 2555 East San Ramon Avenue, Fresno, CA 93740, USA*
[4] *Dipartimento di Scienze Agrarie, Alimentari e Forestali, Università degli Studi di Palermo, Viale delle Scienze, Ed 4, Palermo, Italy*
*Corresponding author. E-mail: grazia.licciardello@crea.gov.it

Summary. Anthracnose caused by *Colletotrichum* spp. is the most damaging olive fruit disease in many countries, including Italy. This disease has been sporadically detected in Sicily, but new agronomic practices can increase risk of olive anthracnose in this region. An etiological study of the disease focused on local olive cultivars growing at the International Olive Germplasm Collection (IOGC) in Villa Zagaria, Enna, Sicily has been undertaken. During 2018 and 2019, 137 *Colletotrichum* strains were isolated from olives. Colony morphology, conidium characteristics, and multilocus sequence analyses aided identification of three species: *C. acutatum* (affecting 70% of symptomatic olives), *C. gloeosporioides,* and *C. cigarro*. Three *C. acutatum* strains (B13-16, P77, and P185), and one strain of each *C. gloeosporioides* (C2.1) and *C. cigarro* (Perg6B) were evaluated for pathogenicity on olive fruits from 11 Sicilian cultivars, known for their high-quality oil. Differences in virulence were detected among strains and their pathogenicity to the cultivars. The *C. acutatum* isolates were more virulent than those of *C. gloeosporioides* or *C. cigarro*. The Sicilian olive cultivars Cavaliera, Carolea, Calatina, and Nocellara del Belice were the most susceptible to the pathogen, while the cultivars Biancolilla and Nocellara Etnea were the most tolerant. Cultivar response under field conditions showed that anthracnose severity and fruit-rot incidence were positively correlated. This is the first report of *C. acutatum* and *C. cigarro* affecting olive trees in Sicily. Control measures for anthracnose depend on accurate characterization of the etiological agents and host cultivar resistance.

Keywords. Pathogenicity, multilocus analyses, cultivar susceptibility, phylogenetic tree.

OPEN ACCESS

Citation: G. Licciardello, J. Moral, M.C. Strano, P. Caruso, M. Sciara, P. Bella, G. Sorrentino, S. Di Silvestro (2022) Characterization of *Colletotrichum* strains associated with olive anthracnose in Sicily. *Phytopathologia Mediterranea* 61(1): 139-151. doi: 10.36253/phyto-13181

Accepted: February 15, 2022

Published: May 13, 2022

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Epaminondas Paplomatas, Agricultural University of Athens, Greece.

ORCID:
GL: 0000-0002-2846-9009
JM: 0000-0002-1153-2809
MCS: 0000-0001-7152-5064
PC: 0000-0001-7153-3953
MS: 0000-0002-0924-1287
PB: 0000-0002-7215-184X
GS: 0000-0002-0153-9337
SDS: 0000-0002-4323-1920

INTRODUCTION

Italy ranks second (after Spain) in olive oil production, with average production of 400,000 tonnes per year (ISTAT, 2021). Olive oil production in

Sicily was 16% of the total Italian yield in 2020 (ISMEA, 2020). In this region, olive trees (*Olea europaea* subsp. *europaea* L.) have been widely cultivated since ancient times, and wild olives are important components of the Mediterranean scrub vegetation. In this part of Italy, due to favorable growing conditions for olives, high intracultivar variations have been accumulated (Muzzalupo *et al.*, 2011). Most olive oils produced in Sicily are extra virgin, with eight recognized as Protected Designation of Origin (PDO) and 28 certified as Protected Geographical Indication (PGI).

Olive anthracnose, caused by *Colletotrichum* spp., is the most damaging olive fruit disease (Moral *et al.,* 2021). These pathogens cause rot and drop of mature drupes, chlorosis and necrosis of leaves, and dieback of twigs and branches (Cacciola *et al.*, 2012). Olive anthracnose epidemics are affected by the autumn rainfall, inoculum density, fruit maturity, and susceptibility of host varieties (Moral and Trapero, 2012). Oil extracted from olives affected by *Colletotrichum* spp. has off-flavours associated with reddish colour and high acidity (Moral *et al.*, 2014; Peres *et al.*, 2021).

Eighteen *Colletotrichum* species have been associated with olive trees (Talhinhas *et al.,* 2018; Moral *et al.,* 2021). These fungi have endophytic to necrotrophic lifestyles and are characterized by a high phenotypic and genotypic diversity (Moral *et al.*, 2009; Talhinhas *et al.*, 2011; Cacciola *et al.*, 2012; Talhinhas and Baroncelli, 2021). The *Colletotrichum* species associated with olive were primarily classified as *C. acutatum* and *C. gloeosporioides* species complexes (Talhinhas *et al.*, 2018). Subsequently, phylogenetic studies have identified numerous species in the *C. acutatum* complex associated with the disease. These include *C. acutatum sensu stricto* (hereafter *C. acutatum*), *C. fioriniae, C. nymphaeae*, *C. simmondsii, C. godetiae,* and *C. rhombiforme* (Mosca *et al.*, 2014; Chattaoui *et al.*, 2016; Talhinhas *et al.*, 2018). Among these species, *C. acutatum* has been described in many Mediterranean countries, including Portugal, Italy, Morocco, Greece, Tunisia, and Egypt (Talhinhas *et al.*, 2005; Rhouma *et al.*, 2010; Embaby *et al.*, 2014; Chattaoui *et al.*, 2016; Msairi *et al.*, 2017; Iliadi *et al.*, 2018).

The *C. gloeosporioides* species complex includes 22 species and one subspecies, although there is little information on the identity of isolates obtained from olive fruit (Weir *et al.*, 2012). Although *C. gloeosporioides* and *C. theobromicola* species complexes have been reported as causal agents of olive anthracnose, they have not been associated with disease outbreaks (Agosteo *et al.*, 2002; Talhinhas *et al.*, 2009). Five additional species (*C. aenigma, C. kahawae* subsp. *cigarro, C. karstii, C. queenslandiicum*, and *C. siamense*) in the *C. boninense* and *C. gloeosporioides* species complexes have also been identified as pathogenic to artificially inoculated ripe olive fruit (Mosca *et al.*, 2014; Schena *et al.*, 2014). *Colletotrichum kahawae* subsp. *kahawae* and subsp. *cigarro* have been separated into two different species, respectively *C. kahawae* and *C. cigarro comb. et stat. nov.* (hereafter *C. cigarro*). This was based on morphological, biochemical, and molecular data (Cabral *et al.*, 2020). Similarly, *C. helleniense* has been described as a new species, phylogenetically close but clearly differentiated from *C. kahawae* (Guarnaccia *et al.,* 2017).

Colletotrichum species have caused several olive anthracnose epidemics in Italy since the 1940s. During the last two decades, the disease has been reported in new olive production areas in southern Italy, mainly caused by *C. godetiae* (syn. *C. clavatum*) (Agosteo *et al.*, 2002; Faedda *et al.*, 2011; Mosca *et al.*, 2014; Schena *et al.*, 2014). Olive anthracnose was first reported in Sicily by Graniti *et al.* (1954). Many years later, *C. gloeosporioides* was associated with the disease in this region (Cacciola *et al.*, 2012). Although many *Colletotrichum* spp. have been identified affecting different plants in Sicily (Faedda *et al.*, 2011; Polizzi *et al.,* 2011, Aiello *et al.*, 2015; Ismail *et al.*, 2015; Guarnaccia *et al.*, 2017), knowledge of the *Colletotrichum* spp. in Sicilian olives is inconsistent and unclear. As well, susceptibility of the local varieties to these fungi is unknown. Increased risk of olive anthracnose is expected because new olive plantations include susceptible foreign cultivars (e.g., 'Arbequina'), and intensive agronomic practices (high plant density and irrigation) (Moral and Trapero, 2012) are increasingly used. It is therefore important to gain knowledge of the main *Colletotrichum* species present in olives in Sicily, and of the susceptibility to anthracnose of local olive varieties.

The objectives of the present study were: i) to accurately identify the main species of *Colletotrichum* causing anthracnose of olive in Sicily, using multilocus phylogenetic analyses; and ii) to characterize the virulence of these fungi, and determine the susceptibility to them of 11 traditional Sicilian olive cultivars.

MATERIALS AND METHODS

Sampling and isolation of fungi

During December 2018 and 2019 anthracnose-affected fruits were collected in the International Olive Germplasm Collection (IOGC) at Villa Zagaria (lat. 37°30'52''N; long. 14°17'46''E), where the incidence of anthracnose was approx. 10%. The IOGC is located close to the Pergusa Lake (c. 650 m above sea level), and

includes 400 olive accessions distributed in four plots: 53 accessions of local (Enna Province) cultivars, 45 accessions from other Sicilian Provinces, 180 from different regions of Italy, and 126 international cultivars. The Enna Province has a dry sub-humid Mediterranean climate (Thornthwaite index) with most rainfall occurring from September to February each year. The olive trees were planted in 2004 and are occasionally treated with copper-based fungicides.

Isolations of *Colletotrichum* spp. were made from olive fruit showing symptoms of anthracnose. Small portions of the mesocarp were removed from affected fruit areas, and were then surface sterilized in a sodium hypochlorite solution (10%) for 30 s. Following three rinses with sterile water, the pieces were plated onto Potato Dextrose Agar (PDA) amended with 100 μg mL^{-1} streptomycin, and were then incubated at 25°C for 10 d. Cultures were also established from fruit surface acervuli onto Petri plates containing PDA.

Colletotrichum cultures on PDA were visually classified into different groups according to their morphological characteristics (Table 1). Single conidium cultures were used for sequencing target genes and pathogenicity tests. All the strains were stored in the CREA - Centro di Ricerca di Olivicoltura, Frutticoltura e Agrumicoltura (Italy, Acireale) culture collection.

Amplification and sequencing of target genes

Partial regions of four loci from eight isolates were amplified from lysed mycelium after heat treatment (100°C for 10 min) (Supplementary Table S1). The primers ITS1 and ITS4 were used to amplify the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA operon, including the 3' end of the first internal transcribed spacer region, the 5.8S rRNA gene, the second ITS region, and the 5' end of the 28S rRNA gene. For each isolate, the partial glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was amplified using primers GDF1 and GDR1 (Guerber *et al.,* 2003). The primers ACT-512F and ACT-783R were used to amplify part of the actin gene (*ACT*), while the partial beta-tubulin (*TUB2*) gene was amplified with primers T1 and Bt-2b (Guarnaccia *et al.*, 2017). As well, a 900 bp intron of the glutamine synthetase (GS) gene was amplified and sequenced from isolate Perg6B using primers GSF1 and GSR1 (Guerber *et al.*, 2003), and submitted to GenBank under the accession number MW053386.

The PCR amplification mixtures and cycling conditions for the four loci were as described by Damm *et al.* (2012). Briefly, the PCRs were performed in a Veriti 96 wells Thermal Cycler (Applied Biosystems) in total volumes each of 25 μL. The ITS, GAPDH, ACT and TUB2 PCR mixtures each contained 1 μL of lysed mycelium, 0.2 μM of each primer, 1 × PCR DreamTaq Green PCR Master Mix (Thermo Scientific Inc.), and 1.26 μL DMSO (not added in the ITS mixture). Amplification conditions constituted an initial denaturation step of 5 min at 94°C, followed by 40 cycles each of 30 s at 94°C, then 30 s at 52°C for GADPH, 55°C for TUB2, 58°C for ACT, or 56°C for ITS, and 30 s at 72°C, and a final elongation step of 7 min at 72°C. The PCR products were enzymatically purified by Exosap-IT™ PCR product clean-up Reagent (Thermofisher Scientific Inc.), and were sequenced in both directions by Microsynth®. Generated DNA sequences were analysed, and consensus sequences were computed using the software MEGA X (Kumar *et al.*, 2018).

Table 1. Strains of *Colletotrichum* from different Sicilian olive cultivars used in the present study.

Isolate	Species	Host cultivar	Isolation matrix
P77	*Colletotrichum acutatum*	Giarraffa	Mesocarp
P185	*C. acutatum*	Vaddarica	Single acervuli
B13-16	*C. acutatum*	Biancolilla nana	Mesocarp
33B	*C. acutatum*	Brandofino	Mesocarp
177B	*C. acutatum*	Zaituna	Single acervuli
237-5	*C. acutatum*	Cipressino	Mesocarp
105-6B	*C. acutatum*	Nocellara del Belice	Mesocarp
105-AC4	*C. acutatum*	Nocellara del Belice	Single acervuli
Perg6B	*C. cigarro*	Biancolilla	Mesocarp
C2.1	*C. gloeosporioides*	Carolea	Mesocarp

Phylogenetic analyses

The new sequences generated in this study were blasted against the NCBI's GenBank nucleotide database to determine the closest sequences for a taxonomic framework. The sequences were aligned using MEGA X (Kumar *et al.*, 2018) and trimmed according to nucleotide length with sequences retrieved from GenBank based on recent studies (Damm *et al.*, 2012; Weir *et al.*, 2012; Guarnaccia *et al.*, 2017; Baroncelli *et al.*, 2017) (Supplementary Table S1). Phylogenetic analyses were carried out individually for each locus (data not shown), and the four loci combined using Bioedit v7.0 to establish isolates at the species level. Two separate analyses were carried out, one for strains belonging to the *C. acutatum* species complex and another for the *C. gloeosporioides* species complex. The multigene analysis of the *C. acutatum* species complex consisted of 47 sequences,

including the outgroup sequence of *C. orchidophilum* (CBS 632.80). Isolates belonging to the *C. gloeosporioides* complex were grouped in a combined phylogeny consisting of 35 sequences, including the outgroup sequence of *C. orchidophilum* (CBS 632.80). A maximum parsimony analysis was performed for each gene, and the multilocus alignment (ITS, *TUB2, GAPDH,* and *ACT*), using MEGA X. Tree length (TL), consistency index (CI), composite index (CI), and Retention Index (RI) were calculated for parsimony. The bootstrap analysis was based on 1000 replications. Sequences generated in this study were deposited in GenBank (Supplementary Table S1).

Pathogenicity and cultivar susceptibility tests on fruit

Pathogenicity tests were carried out using olive fruit of 11 different Sicilian cultivars, and 'Frantoio' used as a resistant control cultivar (Tables 2 and 3). Fruit samples collected at the onset of ripening from healthy olive trees were used, since unripe fruits are unhelpful to discriminate the phenotypic reaction of the olive cultivars against the pathogen (Moral *et al.*, 2008). The fruits were washed, surface sterilized with a 10% solution of commercial bleach (50 g L^{-1} Cl) in sterile water for 1 min. They were then rinsed twice with distilled water, allowed to air dry, and were stored at 4°C in plastic containers until use (less than 7 d). Fruits were inoculated by spraying conidium suspensions of five representative strains of *Colletotrichum* using the methods described by Moral *et al.* (2008). The conidium suspensions were each adjusted to 10^5 conidia mL^{-1}, and sterile water was used as experimental controls. Inoculated and control fruits were incubated in moist chambers (plastic containers, each 22 × 16 × 10 cm, with 100% RH) at 23 ± 2°C under fluorescent lights (12 h photoperiod of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$). There were three replicates (moist chambers) per treatment and ten fruits per replicate. The experiment was conducted twice. Disease severity was assessed every 5 d for 20 d, using a 0 to 5 scale based on symptoms affecting the fruit surfaces where 0 = no visible symptoms, 1 = symptoms affecting less than 25% of the fruit surface, 2 = 25 to 50%, 3 = 51 to 75%, 4 = 76 to 100% of surface affected but with little pathogen sporulation, and 5 = fruit completely rotted showing abundant conidia in gelatinous matrices (soapy fruit) (Moral *et al.*, 2008). Disease index (DI) was calculated using the following formula:

$$DI = (\Sigma n_i \times i)/N$$

where i is the severity score (0 to 5), n_i is the number of fruits with the severity of i, and N is the total number of inoculated fruits.

For each treatment and repetition, the Area Under Disease Progress Curve (AUDPC) was calculated as the area under the DI curve over time, according to Moral *et al.* (2008). Fungi were re-isolated from inoculated fruits onto PDA, and the resulting colonies were compared with the inoculated isolates.

Cultivar responses under field conditions

Fruit-rot incidences of 20 olive cultivars maintained in the IOG Collection at Villa Zagaria were assessed in December 2019. Eighteen cultivars were selected among the IPG Sicilian varieties, and the cultivars Cipressino (susceptible) and Frantoio (resistant) were used as comparisons (Moral *et al.,* 2017). Each cultivar included four replicated olive trees. Olive fruits were harvested at different ripening stages, from green (colour class = 0) to black (colour class = 4), according to Moral *et al.* (2008). The incidence (%) of fruit with unequivocal anthracnose symptoms was quantified in a sample of 300 fruits per cultivar, under laboratory conditions. Disease severity was assessed using the 0 to 5 severity scale described above.

Data analyses

Two-way and one-way ANOVA were performed on AUDPC data, and cultivar means were compared using the Student-Newman-Keuls test at $P \leq 0.01$. A heatmap with dendrograms was displayed using the severity of the disease as dependent variability, to examine similarities between olive cultivars or *Colletotrichum* strains. Disease incidence (%) for each olive cultivar in the orchard trial was compared with the response of the same cultivar in artificial inoculations using the Pearson's correlation test. In addition, a Zar's test of multiple comparisons of these proportions was carried out to examine the effect of the cultivar on the fruit rot incidence (Zar, 2010). Relationships between fruit rot incidence and ripening were analyzed using Pearson's correlation coefficient. Relationship between fruit rot incidence and disease index were assessed using different linear and non-linear models using exponential and potential equations (Campbell and Madden, 1990). The best model was selected based on the statistical significance of the estimated parameters ($P \leq 0.05$), Mallow's Cp statistic, Akaike's information criterion, and the coefficient of determination (R^2). Data were analyzed using Statistix 10 (Analytical Software) and R Studio.

RESULTS

Morphological characteristics of Colletotrichum *spp. colonies*

A total 137 *Colletotrichum*-like colonies were isolated from symptomatic olive fruits and were divided into two groups according to their morphological traits. Ninety-six of the strains (70%) had colonies with regular margins and dense cottony and white or pale salmon aerial mycelium with pinkish spore masses. Within this group, the isolates P77, P185, B13-16, 105B, 177B, 33B, 105-AC4, and 237-5 had conidia that were aseptate, hyaline, and with fusiform ends (Table 1, Figure 1), and these isolates were classified as *C. acutatum* species complex. The second group included 15 strains (10%) characterized by colonies with dark olive-grey mycelium, many orange and black acervuli, and concentric dark circles on the reverse sides (Table 1, Figure 1). Conidia of these strains were aseptate, hyaline, and each slightly constricted in the middle and rounded at each end, and the strains were classified as *C. gloeosporioides* species complex. Among this species complex, the strains Perg6B and C2.1 were selected for further studies. In addition, colonies of *Fusarium lateritium*, *Alternaria* spp., *Phoma foliofoma*, and *Septoria protearum* were occasionally isolated from diseased fruits.

Phylogenetic analyses

Eight representative strains of the two *Colletotrichum* species complexes were selected for further characterization by phylogenetic analyses and taxonomy (Supplementary Table S1). The four phylogenetic trees, derived from single gene sequence alignment for *C. acutatum* and *C. gloeosporioides* species complexes, were topographically similar to those previously reported (Damn *et al.*, 2012; Weir *et al.*, 2012; Baroncelli *et al.*, 2017). These trees confirmed that strains P77, P185, B13-16, 105B, 177B, and 237-5 belong to the *C. acutatum* species complex, while strains Perg6B and C2.1 belong to the *C. gloeosporioides* species complex.

A total of 1469 characters (ITS: 1–469, TUB2: 477–980, GAPDH: 988–1217, ACT: 1225–1469) were analyzed to assess phylogeny of the *C. gloeosporioides* species complex. Of these, 291 were parsimony-informative, 507 were variable and parsimony-uninformative, and 916 were conserved. A maximum of 1000 equally most parsimonious trees were saved (TL = 866, CI = 0.6348, RI = 0.8052, CI = 0.5988). Strain Perg6B clustered within the *C. kahawae/C. cigarro* phylogenetic group. According to nucleotide sequence analysis of the glutamine synthetase gene, isolate Perg6B unequivocally belonged to *C. cigarro*, due to the presence of a 22 bp insertion, which was

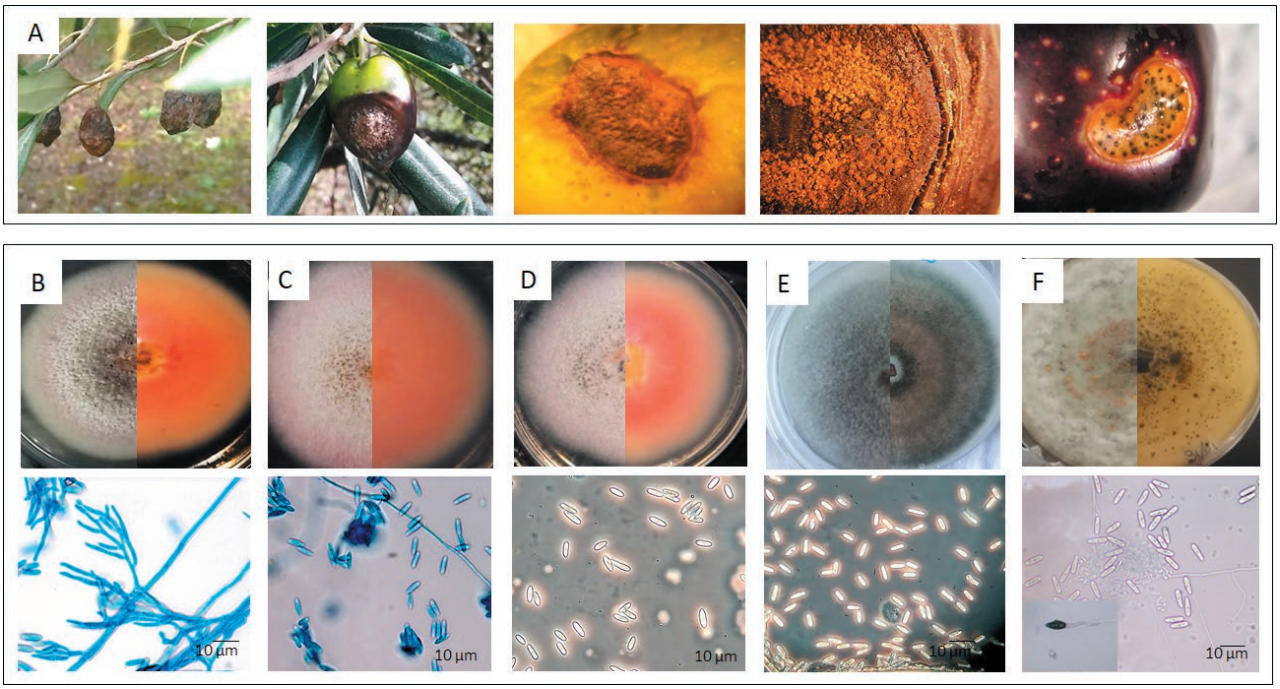

Figure 1. Symptoms of anthracnose (i.e. mummified fruits on the tree, rot, circular sunken lesions) found in olive fruits of the International Olive Germplasm Collection (IOGC) at Villa Zagaria (Sicily, Italy) (A). Above and below views of colony morphology of PDA cultures (top), and respective conidia or conidiophores (bottom) of *Colletotrichum acutatum* P185 (B), *C. acutatum* B13-16 (C), *C. acutatum* P77 (D), *C. gloeosporioides* C2.1 (E), and *C. cigarro* Perg6B (F).

absent in *C. kahawae* isolates. Strain C2.1 was classified as *C. gloeosporioides* (Figure 2).

Regarding the *C. acutatum* species complex, a total of 1321 positions (ITS: 1-407, TUB2: 414-906, GAPDH: 913-1112, and ACT: 1119-1321) were included in the analysis. A total of 163 were parsimony-informative, 318 were variable and parsimony-uninformative, and 978 characters were conserved. A maximum of 1000 equally parsimonious trees were saved (TL = 501; CI = 0.6047; RI = 0.8881; CI = 0.6541). The strains P77, B13-16, P185, 105-6B, 177B, and 237-5 clustered with reference strains of *C. acutatum* (CBS 112996, CBS 112759, CBS 129952) and two Sicilian strains (CBS 142407 and CPC 26987) obtained from *Citrus* (Figure 3).

Pathogenicity on detached fruits

All the inoculated olive fruits developed typical anthracnose symptoms (soapy rot). Two-way ANOVA showed statistically significant differences ($P < 0.001$) between *Colletotrichum* strains and olive cultivars and their interactions. For this reason, differences in cultivar susceptibility were examined for each pathogen strain (Table 2). Fruits of the resistant 'Frantoio' showed slight symptoms on few fruits. In contrast, the first anthracnose symptoms were observed in the fruits of 'Calatina' and 'Carolea' at 5 d after inoculation with the *C. acutatum* isolates.

Fruits of 'Frantoio' were almost completely resistant to the five *Colletotrichum* strains, while among all the other tested cultivars 'Biancolilla', 'Ogliarola messinese', and 'Nocellara messinese' were the most resistant. However, 'Nocellara messinese' developed greater disease severity than 'Vaddarica' when inoculated with isolate Perg6B of *C. cigarro* (Table 2). In contrast, the cultivars Carolea, Cavaliera, and Calatina were the most susceptible, in all the cases showing average AUDPCs > 42. The dendrogram of the heatmap (Figure 4), indicated that the cultivars formed three groups: resistant ('Frantoio'), susceptible ('Nocellara messinese', 'Ogliarola messinese', 'Biancolilla', and 'Vaddarica', 'Santagatese') and highly susceptible ('Nocellara del Belice', 'Carolea', 'Calatina', 'Nocellara etnea', 'Zaituna', 'Cavaliera').

Regarding virulence of the strains, the three *C. acutatum* strains caused a similar severity of symptoms on all the tested cultivars (AUDPC ≈ 40), but greater severity than those caused by *C. gloeosporioides* (AUDPC = 23.1) and *C. cigarro* (AUDPC = 21.7). The three *C. acutatum* strains were grouped according to the heatmap dendrogram, while the other two species were in the same clade (Figure 4). *Colletotrichum* isolates re-isolated on PDA medium from inoculated fruits showed morphological characteristics the same as the inoculated isolates.

Cultivar responses under field conditions

Symptoms on mature fruits occurred as extensive dark necroses, with large orange conidial masses and emerging black acervuli (Figure 1), and these symptoms were also evident in some immature fruits of susceptible cultivars. In green fruits, the rots appeared as small brown sunken lesions. Mummified fruits were also detected, although in small proportion (6%). The correlation between the fruit-rot incidence (%) and the ripening-scale [from 0 (green) to 4 (black)] was statistically significant (Spearman coefficient $r = 0.909$).

Since the disease severity and fruit rot were greater in the ripening fruits comparisons were made between olive cultivars showing the same ripening stage (Table 3). Fruit rot incidence and DI were low in the cultivars with green fruits (ripening class 0) with mean incidence of 0.48% and mean DI of 0.01. In the cultivars with green-yellowish fruits (ripening class = 1), there were significant differences among the cultivars for fruit rot incidence, but not for DI. For the cultivars with fruits at ripening class = 2, the cultivar Nocellara Etnea was more susceptible to *Colletotrichum* spp. than 'Moresca', 'Turdunazza', or 'Zaituna'. For the cultivars showing fruit at ripening class = 3, the cultivar Nocellara del Belice was the most resistant (Table 3). For the cultivars with ripe fruit (colour class = 4), 'Tonda iblea' was more resistant ($P < 0.05$) to the pathogen than 'Cipressino', but only for DI. DI and the fruit rot incidence (%) were significantly correlated ($r = 0.855$; $P < 0.001$).

Although different nonlinear regression models were evaluated for describing the relationship between disease severity and incidence, the best (and selected) model was a linear regression. This regression was forced through the origin because of the biological meaning (Figure 5).

A comparison of results from the pathogenicity tests in controlled conditions and the natural incidence of fruit rots, conducted for the cultivars Santagatese, Ogliarola messinese, Frantoio, Vaddarica, Nocellara messinese, and Calatina which all had the same maturation levels (value of 0-1), showed that there was a statistically significant correlation between these two parameters (Pearson coefficient $r = 0.689$; $P < 0.001$).

DISCUSSION

Olive anthracnose has been rarely reported in Sicily in traditional olive groves, suggesting that agronomic (e.g., cultivar resistance) or climatic conditions are not suitable for disease epidemics (Cacciola *et al.,* 2012). New agronomic practices, including high-density plantations with non-local olive cultivars or new irrigation systems,

can increase the risk of anthracnose in this region of Italy. In the present study, anthracnose incidence was assessed in the Olive Germplasm Collection of Villa Zagaria, which is located in a subhumid area in Enna Province, and which is managed according to traditional agronomic practices.

Ten representative *Colletotrichum* isolates were recovered from approx. 80% of symptomatic olive drupes, and the isolates were identified by phylogenetic and taxonomic analyses, and their pathogenicity on detached fruits was assessed.

For the isolates, colony morphology, conidium characteristics, and multigene phylogenetic analyses identified three main species. *Colletotrichum acutatum* was the most frequently isolated fungus from symptomatic olive fruits, along with a fewer isolates of *C. gloeospori-*

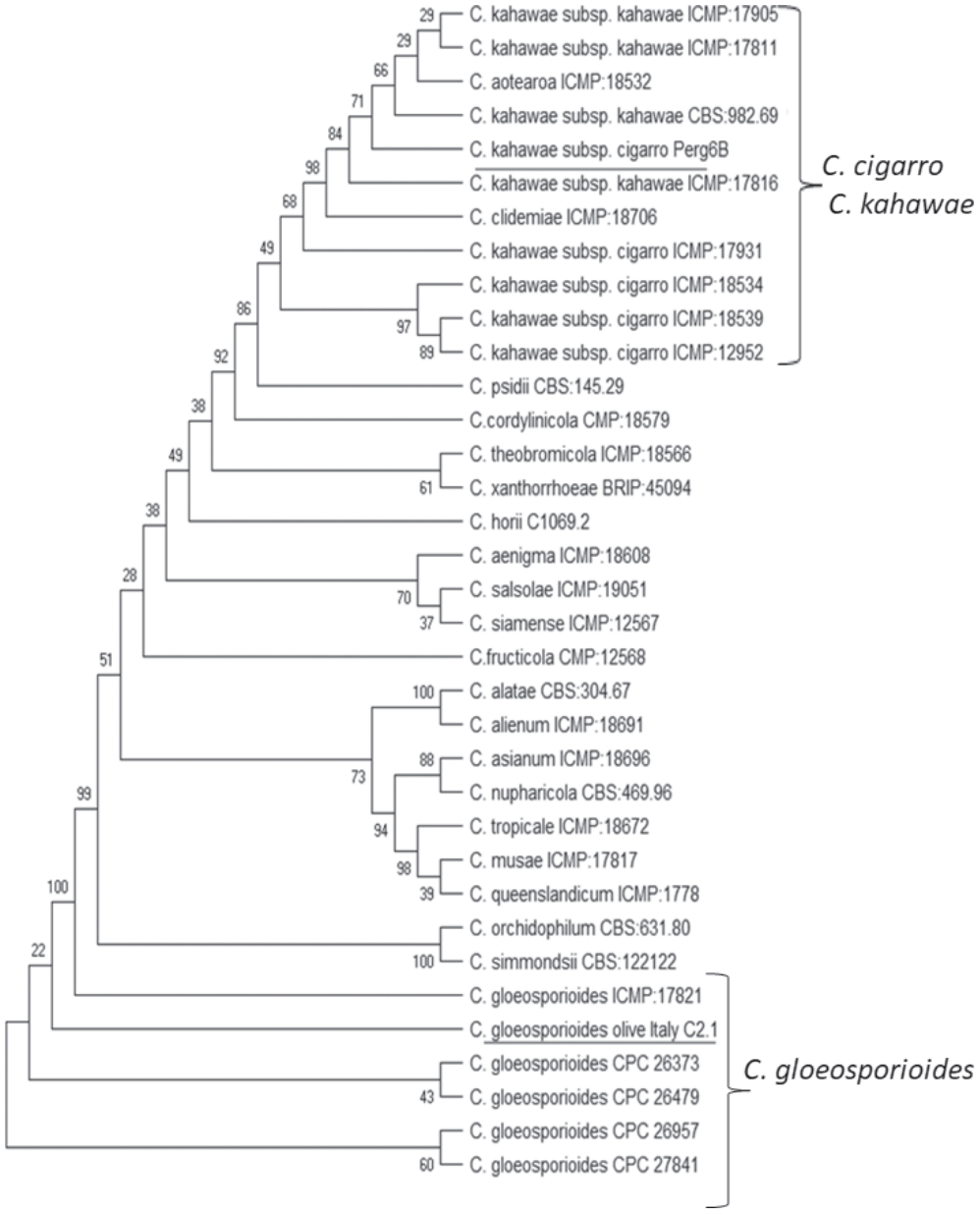

Figure 2. Phylogenetic analysis of the 35 *Colletotrichum gloeosporioides* species complex strains listed in Supplementary Table S1, based on a multilocus concatenated alignment of the ITS, *GAPDH, ACT*, and *TUB2* genes. The evolutionary history was inferred using the Maximum Parsimony method. *Colletotrichum orchidophilum* CBS 632.80 was used as an outgroup. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Strains described for the first time are underlined.

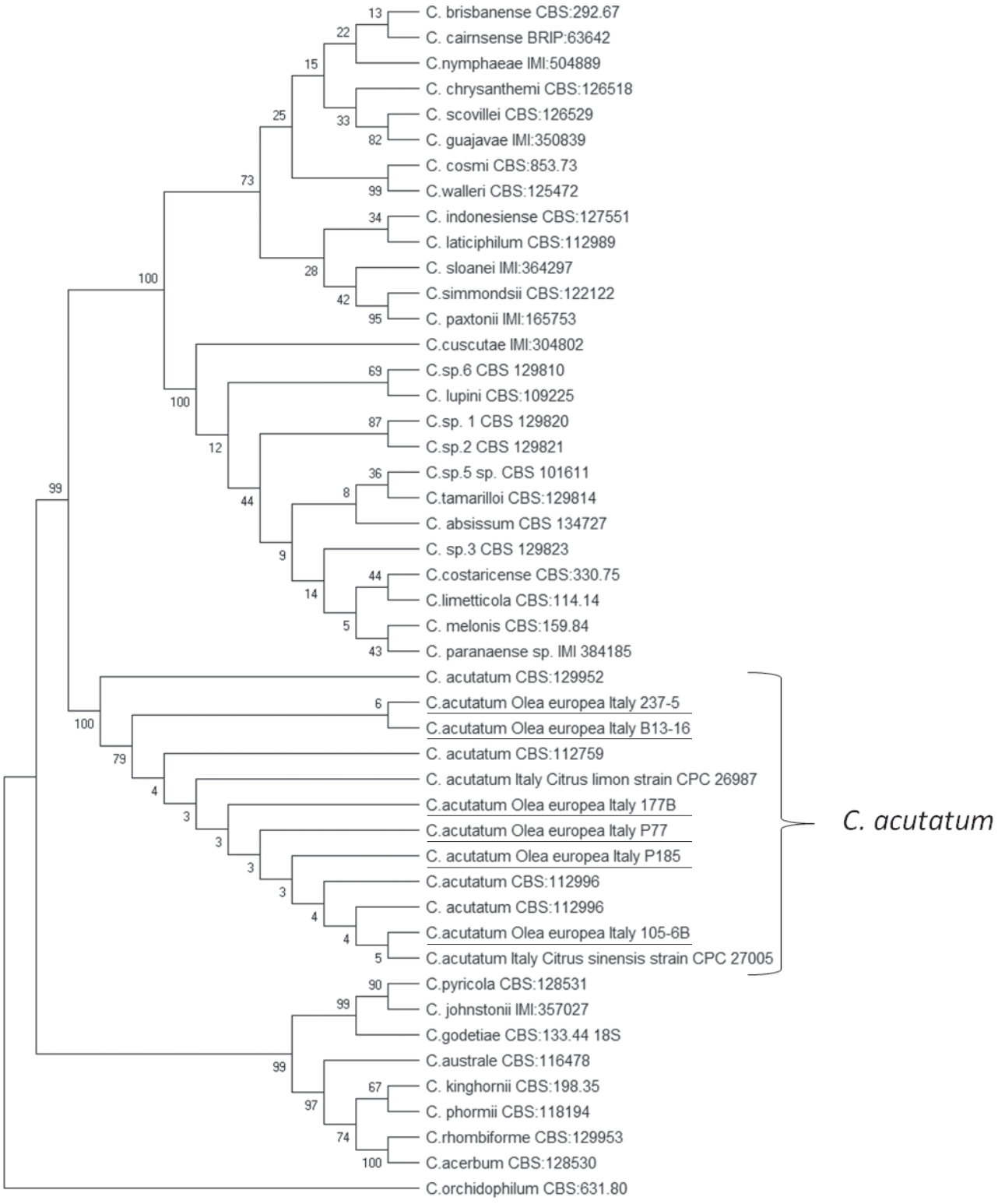

Figure 3. Phylogenetic analysis of the 47 *Colletotrichum acutatum* species complex strains listed in Supplementary Table S1, based on a multilocus concatenated alignment of the ITS, *GAPDH, ACT,* and *TUB2* genes. The evolutionary history was inferred using the Maximum Parsimony method. *Colletotrichum orchidophilum* CBS 632.80 was used as an outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Strains described for the first time in this study are underlined. For strains originating from Italy, the hosts are indicated.

Table 2. Mean Area Under Disease Progress Curves (AUDPCs) for 11 traditional Sicilian olive cultivars and 'Frantoio' (used as resistant control) inoculated with three isolates of *Colletotrichum acutatum* (P185, P77 or B13-16), or one isolate each of *C. gloeosporioides* (C2.1), or *C. cigarro* (Perg6B).

Cultivar	*C. acutatum*			*C. gloeosporioides*	*C. cigarro*	Average
	P185	P77	B13-16	C2.1	Perg6B	
Frantoio	0.8 a*	0.6 a	0.7 a	0.5 a	0.4 a	0.6
Biancolilla	35.8 bc	32.5 b	38.5 cd	14.3 b	14.0 b	27.0
Nocellara messinese	38.0 bc	32.0 b	33.1 b	25.1 c	21.3 cd	29.9
Ogliarola messinese	40.1 c	30.8 b	34.2 bc	22.3 c	17.5 bc	28,9
Santagatese	41.3 c	41.6 c	33.6 bc	23.3 c	13.6 b	30.7
Vaddarica	32.2 b	43.6 c	33.9 bc	29.3 d	14.7 b	30.7
Nocellara etnea	50.2 d	45.2 c	41.4 d	17.0 b	15.1 b	33.7
Nocellara Belice	38.0 bc	41.3 c	32.1 b	35.4 e	34.8 f	36.3
Zaituna	51.2 d	45.2 c	48.2 e	17.0 b	28.0 e	37.9
Cavaliera	53.5 d	52.1 d	50.3 ef	25.0 c	31.9 f	42.5
Carolea	54.1 d	40.3 c	53.5 fg	37.9 e	44.1 g	45.9
Calatina	61.1 e	59.3 e	57.5 g	30.1 d	25.1 de	46.6
Average	41.3	38.7	38.1	23.1	21.7	

*One way ANOVA was performed on AUDPC values. Means within each column accompanied by the same letter are not significantly different ($P < 0.01$), Student-Newman-Keuls test.

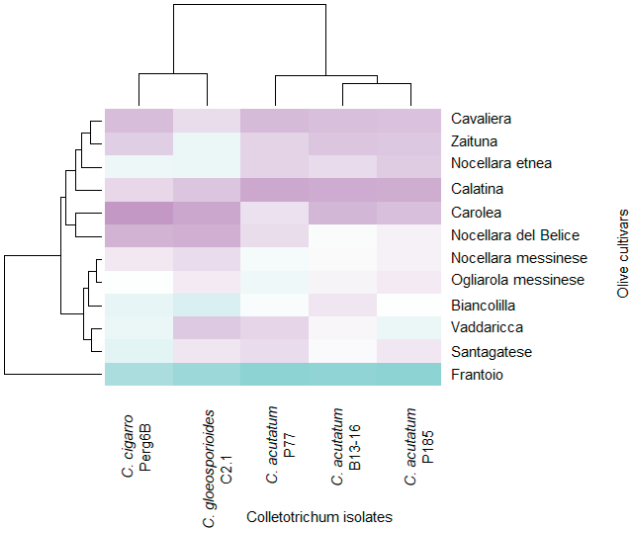

Figure 4. Heatmap and hierarchical clustering of olive fruit susceptibility to anthracnose in 11 Sicilian cultivars, using severity of the disease as the dependent variability to indicate similarity between olive cultivars or *Colletotrichum* strains. The clustering analysis showed three main groups for cultivar susceptibility and two main groups for pathogen strain virulence.

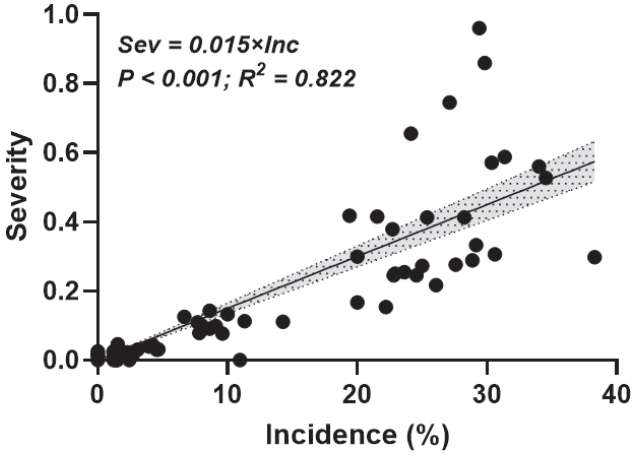

Figure 5. Relationship between fruit rot severity indices and fruit rot incidence (%) for olive fruit affected by *Colletotrichum* spp. in olive trees growing at the International Olive Germplasm Collection (IOGC), Villa Zagaria, Enna, Italy.

oides and *C. cigarro*. While the presence of *C. gloeosporioides* species complex affecting olive fruits was reported by Agosteo *et al.* (2002) in Sicily, the present report is the first for *C. cigarro* and *C. acutatum* associated with olive anthracnose in this region.

Based on ITS sequence analyses, the six strains of *C. acutatum* obtained in the present study are homogenous, and belong to the genotype Acu1, which was prevalent in Calabria, where three genotypes of *C. acutatum* were identified (Mosca *et al.*, 2014). Multilocus sequence analysis showed high homology levels between the isolates and those obtained from *Citrus* in Sicily (Guarnaccia *et al.*, 2017), indicating low diversity and little specificity within the isolates of *C. acutatum*. The data are also

Table 3. Olive fruit ripening classes and disease reactions (mean fruit rot incidence and mean disease index) for 20 selected olive cultivars, naturally infected by *Colletotrichum* spp., in the International Olive Germplasm Collection (IOGC), Villa Zagaria, Enna, Italy.

Cultivar	Ripening class[a]	Fruit-rot (%)[b]	Disease Index[c]
Santagatese	0	0.0 a	0.00 a
Ogliarola Messinese	0	0.0 a	0.00 a
Frantoio Corsini	0	0.0 a	0.00 a
Aitana	0	0.6 a	0.01 a
Minuta	0	1.05 a	0.01 a
Vaddarica	0	1.22 a	0.01 a
Nocellara messinese	1	0.37 a	0.00 a
Calatina	1	1.41 a	0.01 a
Nerba Catanese	1	2.14 b	0.02 a
Cerasuola	1	2.32 b	0.02 a
Moresca	2	9.19 a	0.10 a
Turdunazza	2	9.43 ab	0.10 a
Zaituna	2	9.56 a	0.11 a
Nocellara Etnea	2	25.72 b	0.26 b
Giarraffa	3	31.58 b	0.34 b
Biancolilla	3	24.64 b	0.26 a
Nocellara del Belice	3	22.26 a	0.41 c
Brandofino	3	32.56 b	0.56 d
Tonda iblea	4	21.86 a	0.22 a
Cipressino	4	27.62 a	0.81 b

[a] Fruit ripening was evaluated using a 0 to 4 rating scale (from green to black) (Moral *et al.,* 2017).
[b] Fruit rot incidence was calculated from numbers of infected fruit/total number of fruit. For each ripening scale, means in each column accompanied by different letters are significantly different, Zar's Test for multiple comparisons of proportions (Zar, 2010).
[c] For each ripening scale, cultivar means were compared using the Least Significant Differences (LSD) test at $P = 0.05$.

consistent with the results based on rDNA-ITS sequences, which showed variability up to 4% among *C. acutatum* isolates and indicated they belong to a monophyletic group (Damm *et al.,* 2012; Baroncelli *et al.,* 2017). It is likely that *C. acutatum* was recently introduced in Sicily, as occurred in Portugal and Tunisia (Talhinhas *et al.*, 2009; Chattaoui *et al.*, 2016).

Available data on the diffusion of the *Colletotrichum* population in Italy indicates that *C. godetiae* is dominant. In contrast, the *C. gloeosporioides* species complex was rare (Faedda *et al.*, 2011; Cacciola *et al.*, 2012).

In Portugal, *C. acutatum* is restricted to the Algarve region, whereas *C. nymphaeae*, *C. simmondsii*, and *C. godetiae* were prevalent in other olive-growing regions (Talhinhas *et al.,* 2009). Presence of *C. acutatum* has been described in most of the Mediterranean countries, including Tunisia (Chattaoui *et al.*, 2016), Greece (Iliadi *et al.*, 2018), Morocco (Msairi *et al.*, 2017), and–Egypt (Embaby *et al.*, 2014).

In the present study, analysis of the glutamine synthetase gene allowed the first identification in Sicily of *C. cigarro comb. et stat. nov.* (Cabral *et al.,* 2020) affecting olive fruits. Previously, this species has been detected in Calabria on olive fruits (Schena *et al.,* 2014; Mosca *et al.*, 2014) and mandarin leaves (Perrone *et al.*, 2016), and in Piedmont (northern Italy) from symptomatic leaves and stems of the ornamental plant *Liquidambar styraciflua* (Guarnaccia *et al.*, 2021).

The sporadic detection of *C. gloeosporioides* in the present study confirms the occasional presence of this fungus in Sicilian olives, but it has not been associated with severe disease (Agosteo *et al.*, 2002; Talhinhas *et al.*, 2009; Faedda *et al.*, 2011; Cacciola *et al.*, 2012). However, this species was previously reported affecting sweet orange (*Citrus sinensis*) (Aiello *et al.*, 2015) and mango (*Mangifera indica*) trees in this region (Ismail *et al.*, 2015).

The spread of *C. gloeosporioides* in the Mediterranean region has been at low levels. In Portugal, this fungus occurred sporadically associated with olive anthracnose (3% incidence) in the Algarve region. Only two isolates were identified during a large-scale survey in Northern Tunisia (Chattaoui *et al.*, 2016). However, olive fruits infected by *C. gloeosporioides* species complex have been sporadically reported in Tunisia (Rhouma *et al.*, 2010) and in Morocco (Achbani *et al.*, 2013) and Iran (Sanei and Razavi, 2012).

In Portugal, ITS DNA analysis allowed distinction between two groups of *C. gloeosporioides* isolates, CG-1 and CG-2, differing for only one nucleotide, but with CG-2 isolates associated with greater disease severity than the CG-1 isolates (Talhinhas *et al.*, 2009). The strain C2.1 of *C. gloeosporioides* had the same ITS sequence of other CG-2 olive strains of this species previously described by Schena *et al.* (2014).

Pathogenicity of the *Colletotrichum* strains assessed in the present study was separately evaluated by inoculating detached green fruits of 11 olive cultivars (selected among the most relevant in Sicily) with conidia of isolates of *C. acutatum*, *C. gloeosporioides* and *C. cigarro*. The three strains of *C. acutatum* were the most virulent, and less virulence was demonstrated in the other two species, as has been previously observed (Talhinhas *et al.*, 2011; 2018; Schena *et al.*, 2014; 2017).

The *C. cigarro* isolate was able to infect green detached drupes, reproducing late anthracnose symptoms after 10-15 d (data not shown). Previous inoculation tests on the cultivar Coratina gave no rots on green

fruits and noticeable development of rot on ripening olives (Schena *et al*., 2014), probably due to low susceptibility of this cultivar.

Analysis of symptom evolution under laboratory conditions revealed strong interactions among cultivars and isolates. These require further study. The high susceptibility of the cultivar Nocellara del Belice is also important, as olives of this cultivar were severely diseased when inoculated with *Colletotrichum* strains of low virulence.

Under field evaluations in December, most Sicilian cultivars did not show anthracnose symptoms as previously described for Italian cultivars (Moral *et al*., 2017). The cultivar Cipressino was susceptible in these evaluations, while 'Frantoio' showed well-recognized resistance to the disease (Moral and Trapero, 2009). In contrast, olives of the cultivars Nocellara del Belice, Vaddarica, Santagatese and Oglialora were not affected by the pathogen. This could have been due more to an escape (the fruits were less ripe) than innate resistance (Moral *et al*., 2017). In contrast, all the cultivars, which were evaluated when the fruits were violet-black, developed diseased fruits. This reinforces the recommendation of early harvesting as an effective disease control strategy (Cacciola *et al*., 2012; Moral and Trapero, 2012). Some inconsistencies between the field and laboratory evaluations were observed on the cultivars Calatina and Zaituna, which could be due to the pathogen population or to fruit maturity. The present study is the first to examine the relationship between disease severity and olive fruit rot incidence. In general, both variables were positive and highly correlated, reinforcing the concept that cultivar resistance can be evaluated only according to the incidence of affected fruit (Moral and Trapero, 2012).

In conclusion, the present study has shown that *C. acutatum* is present in olive groves in Sicily. According to DNA sequence analyses, the population is likely to be homogenous, and does not differ from strains isolated from other hosts (i.e., *Citrus*). Since climatic conditions in some olive producing areas in Sicily are not favourable for anthracnose development, in the short term, the risk of disease remains low for most cultivars as they are not susceptible until they ripen. Furthermore, other pests, such as the olive fruit fly (*Bactrocera oleae*), could affect quality of Sicilian olive oils. Climate changes, adaptation to new hosts, or cross-infection events by *Colletotrichum* among different hosts, could lead to re-emergence of olive anthracnose in Sicily, as has occurred for diffusion of these pathogens in the northern hemisphere (Talhinhas *et al*., 2009; Mosca *et al*., 2014). Further surveys are required to classify the field performance of Sicilian cultivars, and to determine interactions among cultivars and isolates, and define pathogen phenotype dynamics.

ACKNOWLEDGEMENTS

This research was financially supported by SALVAOLIVI project (MIPAAFT, DM 59 del 10/01/2018). J.M. is also supported by the EPIDEMIOLIVE project (PID2020-117550RA-I00) funded by the Spanish government (MICIN). The authors thank Dr Andrea Scoto, Dr Antonio Aveni and Libero Consorzio Comunale di Enna for the use of the experimental orchard of IOGC Villa Zagaria.

LITERATURE CITED

Achbani E.A., Benbouazza A., Douira A., 2013. First report of olive Anthracnose, caused by *Colletotrichum gloeosporioides*, in Morocco. *Atlas Journal of Biology* 2: 171–174.

Agosteo G.E., Magnano di San Lio G., Cacciola S.O., Frisullo S., 2002. Characterisation of the causal agent of olive Anthracnose in Southern Italy. *Acta Horticulturae* 586: 713–716.

Aiello D., Carrieri R., Guarnaccia V., Vitale A., Lahoz E., Polizzi, G., 2015. Characterization and pathogenicity of *Colletotrichum gloeosporioides* and *C. karstii* causing preharvest disease on *Citrus sinensis* in Italy. *Journal of Phytopathology* 163:168–177.

Baroncelli R., Talhinhas P., Pensec F., Sukno S.A., Le Floch G., Thon, M., 2017. The *Colletotrichum acutatum* species complex as a model system to study evolution and host specialization in plant pathogens. *Frontiers in Microbiology* 8: 2001.

Cabral A., Azinheira H.G., Talhinhas P., Batista D., Ramos A.P., Silva M.D.C., Oliveira H., Várzea V., 2020. Pathological, morphological, cytogenomic, biochemical and molecular data support the distinction between *Colletotrichum cigarro comb. et stat. nov.* and *Colletotrichum kahawae*. *Plants* 9: 502.

Cacciola S.O., Faedda R., Sinatra F., Agosteo G.E., Schena L., … Magnano di San Lio G., 2012. Olive Anthracnose. *Journal of Plant Pathology* 94: 29–44.

Campbell C. L., and Madden, L.V., 1990. *Introduction to Plant Disease Epidemiology*. John Wiley & Sons, New York, USA, pp. XVII + 532 pp.

Cannon P.F., Damm U., Johnston P.R., Weir B.S., 2012. *Colletotrichum* – current status and future directions. *Studies in Mycology* 73: 181–213.

Chattaoui M., Raya M.C., Bouri M., Moral J., Perez-Rodriguez M.,…Rhouma A., 2016. Characterization of a

Colletotrichum population causing Anthracnose disease on olive in northern Tunisia. *Journal of Applied Microbiol*ogy 120: 1368–1381.

Damm U., Cannon P.F., Woundenberg J.H.C., Crous P.W., 2012. The *Colletotrichum acutatum* species complex. *Studies in Mycology* 73: 27–113.

Embaby E.M., Haggag L.F., Abdel-Monem M.O., El-Sayed T.I., Al-Awam L.R., 2014. Anthracnose disease (*Colletotrichum* sp.) affecting olive fruit quality and its control in Egypt. *Journal of Agricultural Technology* 10: 1289-1306.

Faedda R., Agosteo G.E., Schena L., Mosca S., Frisullo S., … Cacciola S.O., 2011. *Colletotrichum clavatum* sp. nov. identified as the causal agent of olive Anthracnose in Italy. *Phytopathologia Mediterranea* 50: 283–302.

Graniti A., 1954. La "lebbra" delle olive in Sicilia. *Olivicoltura* 9: 1–5.

Guarnaccia V., Groenewald J.Z., Polizzi G., Crous, P.W., 2017. High species diversity in *Colletotrichum* associated with citrus diseases in Europe. *Persoonia* 39: 32–50.

Guarnaccia V., Martino I., Gilardi G., Garibaldi A., Gullino M.L., 2021. *Colletotrichum* spp. causing anthracnose on ornamental plants in northern Italy. *Journal of Plant Pathology* 103: 127–137.

Guerber J.C., Liu B., Correll J.C., Johnston P.R., 2003. Characterization of diversity in *Colletotrichum acutatum* sensu lato by sequence analysis of two gene introns, mtDNA and intron RFLPs, and mating compatibility. *Mycologia* 95: 872–895.

Iliadi M.K., Tjamos E.C., Antoniou P.P., Tsitsigiannis D.I., 2018. First report of *Colletotrichum acutatum* causing Anthracnose on olives in Greece. *Plant Disease* 102: 820.

Ismail A.M., Cirvilleri G., Yaseen T., Epifani F., Perrone G., Polizzi G., 2015. Characterisation of *Colletotrichum* species causing Anthracnose disease of mango in Italy. *Journal of Plant Pathology* 97: 167–171.

ISMEA—Institute of Services for the Agricultural and Food Market. Scheda di Settore Olio d'Oliva 2020. Available online: https://www.ismeamercati.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/3523 (accessed on 20 June 2021).

ISTAT—Italian National Institute of Statistics. Cultivations: Grapes, Wine, Olives, Oil. Available online: http://dati.istat.it (accessed on 22 January 2021).

Kumar S., Stecher G., Li M., Knyaz C., Tamura K., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution* 35: 1547–1549.

Moral J., Trapero A., 2012. Mummified fruit as a source of inoculum and disease dynamics of olive Anthracnose caused by *Colletotrichum* spp. *Phytopathology* 102: 982–989.

Moral J., Bouhmidi K., Trapero A., 2008. Influence of fruit maturity, cultivar susceptibilitity, and inoculation method on infection of olive fruit by *Colletotrichum acutatum*. *Plant Disease* 92: 1421–1426.

Moral J., Oliveira R., Trapero A., 2009. Elucidation of the disease cycle of olive Anthracnose caused by *Colletotrichum acutatum*. *Phytopathology* 99: 548–556.

Moral J., Xaviér C., Roca L.F., Romero J., Moreda W., Trapero A., 2014. La Antracnosis del olivo y su efecto en la calidad del aceite. *Grasas y Aceites* 65: e028.

Moral J., Xaviér C.J., Viruega J.R., Roca L.F., Caballero J., Trapero A., 2017. Variability in Susceptibility to Anthracnose in the World Collection of Olive Cultivars of Cordoba (Spain). *Frontiers in Plant Science* 8: 1892.

Moral J., Agustí-Brisach C., Raya M.C., Jurado-Bello J., López-Moral, A., …Trapero, A., 2021. Diversity of *Colletotrichum* species associated with olive Anthracnose worldwide. *Journal of Fungi* 7: 741.

Mosca S., Li Destri Nicosia M.G., Cacciola S.O., Schena L., 2014. Molecular analysis of *Colletotrichum* species in the carposphere and phyllosphere of olive. *PLoS One* 9: e114031.

Msairi S., Chliyeh M., Rhimini Y., Selmaoui K., Mouria A., Touhami A.O., Benkirane R., Douira A., 2017. First Report on *Colletotrichum acutatum* of Olives in Morocco. *Annual Research and Review in Biology* 16: 1–8.

Muzzalupo I., Chiappetta A., Stabile G., Bucci C., Perri E., 2011. Intra-varietal difference of "Carolea" olive assessed by molecular markers. *Acta Horticulture* 918: 771–776.

Peres F., Talhinhas P., Afonso H., Alegre H., Oliveira H., Ferreira-Dias S., 2021. Olive oils from fruits infected with different Anthracnose pathogens show sensory defects earlier than chemical degradation. *Agronomy* 11: 1041.

Perrone G., Magistà D., Ismail A.M., 2016. First report of *Colletotrichum kahawae* subsp. *ciggaro* on mandarin in Italy. *Journal of Plant Pathology* 98: 12.

Polizzi G., Aiello D., Guarnaccia V., Vitale A., Perrone, G., Stea G., 2011. First report of damping-off on strawberry tree caused by *Colletotrichum acutatum* and *C. simmondsii* in Italy. *Plant Disease* 95: 1588.

Rhouma A., Triki M.A., Msallem M., 2010. First report of olive Anthracnose caused by *Colletotrichum gloeosporioides* in Tunisia. *Phytopathologia Mediterranea* 49: 95–98.

Sanei S.J., Razavi S.E, 2012. Survey of olive fungal disease in north of Iran. *Annual Review and Research in Biology* 2: 27–36.

Schena L., Mosca S., Cacciola S., Faedda R., Sanzani S., … Magnano di San Lio G., 2014. Species of the *Colletotrichum gloeosporioides* and *C. boninense* complexes associated with olive Anthracnose. *Plant Pathology* 63: 437–446.

Schena L., Abdelfattah A., Mosca S., Li Destri Nicosia M.G., Agosteo G.E., Cacciola S.O., 2017. Quantitative detection of *Colletotrichum godetiae* and *C. acutatum sensu stricto* in the phyllosphere and carposphere of olive during four phenological phases. *European Journal of Plant Pathology* 149: 337–347.

Talhinhas P., Baroncelli R., 2021. *Colletotrichum* species and complexes: geographic distribution, host range and conservation status. *Fungal Diversity.* https://doi.org/10.1007/s13225-021-00491-9.

Talhinhas P., Sreenivasaprasad S., Neves-Martins J., Oliveira, H., 2005. Molecular and phenotypic analyses reveal the association of diverse *Colletotrichum acutatum* groups and a low level of *C. gloeosporioides* with olive Anthracnose. *Applied and Environmental Microbiology* 71: 2987–2998.

Talhinhas P., Neves-Martins J., Oliveira H., Sreenivasaprasad S., 2009. The distinctive population structure of *Colletotrichum* species associated with olive Anthracnose in the Algarve region of Portugal reflects a host-pathogen diversity hot spot. *FEMS Microbiology Letters* 296: 31–38.

Talhinhas P., Mota-Capitao C., Martins S., Ramos A.P., Neves-Martins J., Oliveira H., 2011 Epidemiology, histopathology and aetiology of olive Anthracnose caused by *Colletotrichum acutatum* and *C. gloeosporioides* in Portugal. *Plant Pathology* 60: 483–495.

Talhinhas P., Loureiro A., Oliveira H., 2018. Olive Anthracnose: a yield-and oil quality-degrading disease caused by several species of *Colletotrichum* that differ in virulence, host preference and geographical distribution. *Molecular Plant Pathology* 19: 1797–1807.

Weir B.S., Johnston P.R., Damm U., 2012. The *Colletotrichum gloeosporioides* species complex. *Studies in Mycology* 73: 115–180.

Zar J.H., 2010. *Biostatistical Analysis*. 5th ed. Pearson Education India, New Delhi, India, 944 pp.