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DIVERSITY OF BOTRYOSPHAERIACEAE SPECIES ON MEDITERRANEAN AND TROPICAL PLANTS

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Index

1. Introduction 1

1.1 References 10

2. Experimental part 1: Diversity of Botryosphaeriaceae species associated with canker and dieback on avocado (Persea americana) in Italy 22

2.1 Introduction 23

2.2 Materials and methods 25

2.2.1 Field surveys and fungal isolation 25
2.2.2 Morphological and culture characters of isolates ... 26
2.2.3 DNA extraction and PCR analyses 27
2.2.4 Phylogeny 27
2.2.5 Pathogenicity test 34

2.3 Results 34

2.3.1 Field surveys and fungal isolations 35
2.3.2 Phylogeny 38
2.3.3 Morphological and cultural characteristic of the isolates 40
2.3.4 Pathogenicity test 41

2.4 Discussion 44

2.5 References 48

3. Experimental part 2: A New Disease for Europe of Ficus microcarpa Caused by Botryosphaeriaceae Species 58

3.1 Introduction 59

3.2 Materials and methods 61

3.2.1 Surveys and fungal isolation 61
3.2.2 Morphological and Molecular Characterization 61
3.2.3 Phylogenetic Analysis 63
3.2.4 Pathogenicity test 64

3.3 Results 65

3.3.1 Surveys and Fungal Isolations 65
3.3.2 Morphological Characterization and Phylogenetic Analysis... 66
3.3.3 Pathogenicity test 74

3.4 Discussion 76

3.5 References 81

4. Experimental part 3: Lasiodiplodia citricola, a new causal agent of Acacia spp. dieback 89

4.1 New disease report 90

4.2 References 95

5. Other research activities: Neopestalotiopsis siciliana sp. nov. and N. rosae Causing Stem Lesion and Dieback on Avocado Plants in Italy.. 96

5.1 Introduction.. 97

5.2 Materials and Methods 98

5.2.1 Isolation and Morphological Characterization 98
5.2.2 DNA Extraction, PCR, and Phylogenetic Analysis... 100
5.2.3 Pathogenicity Test 108
5.2.4 Data Analysis... 109

5.3 Results 109

5.3.1 Isolation and Morphological Characterization 110
5.3.2 Phylogenetic Analysis 113
5.3.3 Pathogenicity Test 116
5.3.4 Morphological Description of *Neopestalotiopsis rosae*

Isolates from Avocado 117

5.4 Taxonomy 121

5.5 Discussion 124

5.6 Conclusions 127

5.7 References 128

6. Other research activities: First Report of Rosellinia necatrix Causing White Root Rot on Avocado in Italy 137

6.1 References 141

7. Other research activities: Fusarium nirenbergiae (Fusarium oxysporum Species Complex) Causing the Wilting of Passion Fruit in Italy 142

7.1 Introduction 143

7.2 Materials and methods 144

7.2.1 Field Survey, Isolations and Morphological Characterization 144

7.2.2 Molecular Characterization and Phylogeny 145

7.2.3 Pathogenicity Tests 150

7.3 Results 150

7.4 Discussion and conclusion 154

7.5 References 156

8. Other research activities: Unusual Stylar-End Breakdown and Sour Rot on Key Lime (Citrus aurantiifolia) in Pre-Harvest Condition in Italy 160

8.1 Introduction 161

8.2 Materials and Methods 162

8.2.1 Isolation 162

8.2.2 Morphological and Molecular Characterization 162
8.2.3 Pathogenicity Test 163
8.3 Results 164
8.4 Discussion 168
8.5 Conclusions 171
8.6 References 172
9. General conclusion 175
10. Annexes: Scientific Curriculum 178
10.1 Research and professional experience 178
10.2 Education and Professional qualifications 178
10.3 Scientific contributions 179

Research highlights

- *Botryosphaeriaceae* is an Ascomycetous fungal family belonging to the Botryosphaeriales order.
- These fungi are considering one of the most polyphagous groups affecting a wide variety of plants, including fruit, forest, and ornamental crops.
- They are able to persist as endophytes in healthy trees and saprophytes in dead tissue. Besides they can also survive as latent pathogens for long time and cause disease when the host is under stress.
- Different organs are susceptible to *Botryosphaeriaceae* fungi and are usually associated with canker symptoms, but also leaf spots, twig blight, dieback, internal discoloration, and general wilting.
- Climate-change affects the dynamics of fungal populations and contributes to additional stress or pressure on plants.
- Traditional and innovative crops, such as Tropical plants, have important economic value and represent a substantial income for the Sicilian production.
- *Botryosphaeriaceae* represents a serious risk for those crops, from nursery to open field (urban area included).
- Surveys need to be conducted on avocado because nowadays represents an important economic source for the Sicilian industry.
- Meanwhile, investigation need to be conducted on ornamental trees, that are under investigate and represent a large source of *Botryosphaeriaceae* inoculum.
- Investigations and characterization of the etiological agents causing disease symptoms on those crops, in various area of the Island, are useful to understand the phytopathological status.
- Further study will be needed to elucidate the epidemiology, the latent phase, and the control strategies of the recovered fungi.

Abstract

Botryospheriaceae are important fungi distributed worldwide and able to persist as endophytes, saprophytes, and latent pathogens. In the recent years have aroused great interested due their capability to cause disease when the host is under stress conditions and especially because climate-change positively affect the distribution and virulence of some species. Is well known that climate is one of the main factors affecting the distribution of these fungi and nowadays *Botryosphaeriaceae* represent a serious threat for agricultural, ornamental and forestry ecosystem. There are many reports of *Botryosphaeriaceae* affecting agricultural crops in Sicily (Italy). Innovative and ornamental crops, emerging and widely diffused in the Island, respectively, are still under studied and subjected to *Botryosphaeriaceae* infections. For these reasons we decided to conduct research on those crops. Among them, avocado (*Persea americana*) is an important crop which is increasing its production area year by year and represent a serious source for Sicilian industry. We have conducted surveys in the main Sicilian avocado orchards in order to highlight the diversity of the *Botryosphaeriaceae* species that threatened this host diffusion. At the same time ornamental crops in the urban area and in nursery, that are under investigated and represent an important inoculum source, showed high disease incidence imputable to *Botryosphaeriaceae* infection. In particular, we have conducted surveys on Indian laurel-leaf fig (*Ficus microcarpa*) in the main urban area, and on different *Acacia* sp. growth in a nursery in Milazzo (Sicily). The present research project aimed to elucidate the diversity of the *Botryosphaeriaceae* species in avocado, Indian laurel-leaf fig, and *Acacia* sp. Results of the morphological and molecular characterization revealed presence of different *Botryosphaeriaceae* species and pathogenicity test confirm the capability of the recovered pathogens to cause diseases.

Keywords: *Botryosphaeriaceae*, Mediterranean crops, Tropical crops,

ornamental crops, fungal disease, morphological characterization, phylogeny, pathogenicity test.

Riassunto

Le *Botryosphaeriaceae* sono importanti funghi diffusi in tutto il mondo e in grado di persistere come endofiti, saprofiti e patogeni latenti. Negli ultimi anni hanno suscitato grande interesse a causa della loro capacità di causare malattie quando l'ospite si trova in condizioni di stress, e soprattutto perché il cambiamento climatico influenza positivamente la distribuzione e la virulenza di alcune specie. È ben noto che il clima è uno dei principali fattori che influenzano la diffusione di questi funghi, e attualmente le *Botryosphaeriaceae* rappresentano una seria minaccia per gli ecosistemi agricoli, ornamentali e forestali. Ci sono numerose segnalazioni di *Botryosphaeriaceae* che colpiscono le colture agricole in Sicilia (Italia). Colture innovative e ornamentali, emergenti e ampiamente diffuse nell'isola, sono ancora poco indagate e soggette ad infezioni di *Botryosphaeriaceae*. Per questi motivi abbiamo deciso di condurre ricerche su queste colture. Tra queste, l'avocado (*Persea americana*) è una importante coltura che sta aumentando la sua area di produzione di anno in anno e rappresenta una importante risorsa per l'industria siciliana. Abbiamo condotto indagini nei principali areali di coltivazione di avocado siciliano al fine di mettere in luce la diversità delle specie di *Botryosphaeriaceae* che minacciano la diffusione di questo ospite. Allo stesso tempo, le colture ornamentali nelle aree urbane e nei vivai, che sono poco studiate e rappresentano una importante fonte di inoculo, mostravano un'alta incidenza di malattie imputabili ad infezioni di *Botryosphaeriaceae*. In particolare, abbiamo condotto indagini su *Ficus microcarpa* nelle principali aree urbane, e su diverse specie di *Acacia* in un vivaio (Sicilia). Il presente progetto di ricerca aveva lo scopo di mettere in luce la diversità delle specie di *Botryosphaeriaceae* in avocado, *Ficus* e *Acacia* spp. I risultati della caratterizzazione morfologica e molecolare hanno rivelato la presenza di diverse specie di *Botryosphaeriaceae*, e i test di patogenicità hanno confermato la capacità dei patogeni rinvenuti di causare malattia.

Parole chiave: *Botryosphaeriaceae*, colture mediterranee, colture tropicali, colture ornamentali, malattie fungine, caratterizzazione morfologica, filogenesi, test di patogenicità.

1. Introduction

The *Botryosphaeriaceae* Theissen & Sydow are a large family of ascomycetous fungi subjected to several taxonomic revision carried out over the years. This fungal family was described for the first time in 1918 by Theissen & Sydow as a sub-family of *Pseudosphaeriaceae*. The *Pseudosphaeriaceae* were considered by Theissen (1916) in the order Myriangiales while a year later Theissen and Sydow (1917) thought it more appropriate to unite the *Pseudosphaeriaceae* with the *Dothideaceae* (Luttrell, 1951). Theissen and Sydow (1918) erected the sub-class of the Dothideinae and included the order Pseudosphaeriales, the family *Botryosphaeriaceae* and the genus *Botryosphaeria* within this sub-class. Petrak (1923) rejected Theissen and Sydow (1918) classification and placed *Botryosphaeria* in the sub-family *Pseudosphaerieae* within the *Pleosporaceae*, order Sphaeriales. In 1928, Miller noted significant distinctions among species in the Sphaeriales group, which are identified by the presence of authentic perithecial ascomata and paraphyses. On the other hand, species classified in the Dothideales category are characterized by ascostromatic ascomata without paraphyses. For those reasons, Miller, suggested that *Botryosphaeria* species be appropriately reclassified within the Dothideales due to their absence of authentic perithecial walls. In 1938, Miller conducting investigation regarding the morphological characteristic, with particular attention to the perithecial wall and the development of the tissue in the centrum. He observed that the taxa allocated to the Spheriales had true perithecial ascomata and paraphyses, in contrast with to the Dothideales that had ascostromatic ascomata lacking paraphyses. Therefore, he maintained he maintained *Botryosphaeria* in in the family *Pseudosphaeriaceae* and placed the genus in the order Pseudosphaeriales. Subsequentely. In 1955, Luttrell replace the name Pseudosphaeriales in Pleosporales and *Botryosphaeria* was accomodate in Pleosporales. Barr (1972,

1976) passed over specimens of *B. dothidea* characterised by interthecial tissues, and classified *Botryosphaeria* in the Dothideales. Later, the same author (Barr, 1979) acknowledged that *Botryosphaeria* species had a typical centrum of the Pleosporales and accommodate *Botryosphaeria* in this order. In 1975 (von Arx and Müller), 1981 and 1987 (von Arx) the authors observed the presence of unrelated genera and the overlap of taxonomic characters used to separate the orders in the classification proposed by Luttrell (1955, 1973) and Barr (1972, 1987). Moreover, von Arx and Müller (1975) did not support the placement of *Guignardia* and *Botryosphaeria* in distinct orders, Dothideales and Pleosporales, respectively. For these reasons the authors (von Arx and Müller, 1975) concluded that *Guignardia* and *Botryosphaeria* should be combined in Dothideales and placed all bitunicate ascomycetes in the single order Dothideales, including *Botryosphaeriaceae*. A few years later, in 1984, Sivanesan placed *Botryosphaeria* and *Guignardia* into Dothideales, while Hawksworth *et al*. (1995) described *Botryosphaeria* under *Botryosphaeriaceae* and *Guignardia* under *Mycosphaerellaceae* both in Dothideales order. Traditionally, researchers have been used morphological characters for species identification, and the taxonomy of this fungal family have been confused for many years. In particular, the difficulty of finding teleomorphs in nature or obtaining them under laboratory conditions, has forced the identification of these fungi to be primarily based on anamorphic characters. Therefore, species differentiation based only on anamorphic characters has not been simplified as a result of the fact that different species within the same genera often have overlapping morphological features (Pavlic *et al*., 2009; Slippers *et al*., 2004). Fortunately, the introduction of molecular tools, like DNA sequencing and phylogenetic analyses, helped to clarifying the confused taxonomic status of the *Botryosphaeriaceae*. During the years, several taxonomic studies were carried out using different nuclear and mithocondrial rDNA genes. Research

conducting during these years yielded taxonomic confusion and did not afford the clarification of taxonomic position of the family into Dothideales or Pleosporales (Berbee, 1996; Silva-Hanlin and Hanlin, 1999; Lumbsch *et al*., 2000; Lutzoni *et al*., 2004). In 2006 Schoch *et al*. developed a multigene phylogeny based on SSU, 28S rRNA gene (LSU), translation elongation factor 1-alpha (*tef1*-α) and RNA polymerase second largest subunit (*rpb2*) and reveled that *Botryosphaeria* and *Guignardia* clustered in a separate clade that could not be associated to any other order. For this reason, they introduced the new order Botryosphaeriales Schoch, Crous & Shoemaker in order to accommodate the single family of *Botryosphaeriaceae*. In the same year, Crous *et al*. (2006) split *Botryosphaeria* into ten genera. Following consecutive studies, the families *Planistromellaceae* (Minnis *et al*., 2012), *Phyllostictaceae* (Wikee *et al*., 2013), *Aplosporellaceae*, *Melanopsaceae*, *Saccharataceae* (Slippers *et al*., 2013), *Septorioideaceae* (Wyka and Broders, 2016), *Endomelanconiopsisaceae*, and *Pseudofusicoccumaceae* (Yang *et al*., 2017) were recognized in Botryosphaeriales. Slippers *et al*. (2013) reviewed the historical developments of species identification and taxonomy of the order and highlighted that the biology of these fungi is still not-well understood. Few years later, Phillips *et al*. (2019) decided to re-assess families in Botryosphaeriales based on ITS and LSU sequence data and accepted six families: *Aplosporellaceae*, *Botryosphaeriaceae*, *Melanopsaceae*, *Phyllostictaceae*, *Planistromellaceae* and *Saccharataceae*. *Endomelanconiopsisaceae*, *Pseudofusicoccumaceae* and *Septorioideaceae* were reduced to synonymy under existing families. Recent taxonomic study by Zhang *et al*. (2021) clarified the identity of numerous isolates that lacked latin binomials or had been deposited under incorrect names. The authors also provide a solid foundation for more in-depth future studies on taxa in that order. They showed that sequence of *tef1*-α, *tub2* and *rpb2* genes proved to be the most reliable

markers and the combination of four candidate barcode (ITS, *tef1*-α, *tub2* and *rpb2*) provide a reliable solution. Currently, there are 24 well defined genera, with more than 200 species, among them the four largest being to *Lasiodiplodia*, *Dothiorella*, *Neofusicoccum*, and *Diplodia* (Burgess *et al*., 2019).

Fungi belonging to *Botryosphaeriaceae* display ascospores (hyaline or colored) within bitunicate and clavate asci produced inside uni-to multilocular ascomata that can occur singly or in clusters. Conidia, usually hyaline, can be narrow (*Fusicoccum*-like) or wide (*Diplodia*-like) (Moral *et al*., 2019). Pycnidia are commonly found embedded in diseased woody parts of host tissue as well as in wood debries (van Niekerk *et al*., 2004; Úrbez-Torres *et al*., 2006) and under humid conditions, produce conidia that are generally exuded in gelatinous matrices forming cirrhi (Michailides and Morgan, 1993; Phillips, 2002). These fungal species are distributed in all geographical and climatic diversified area of the world and are capable to cause disease on woody and herbaceous plants. Although some species have been detected in herbaceous plants, *Botryosphaeriaceae* typically cause disease on woody hosts. Some examples are disease on Mediterranean crops such as C*itrus* (Bezerra *et al*., 2021; Polizzi *et al*., 2009; Vakalounakis *et al*., 2019), olive tree (Brunetti *et al*., 2022; Manetti *et al*., 2023; Moral *et al*., 2017), grapevine (Burruano *et al*., 2008; Guerin-Dubrana *et al*., 2019; Linaldeddu *et al*., 2015), pistachio (Gusella *et al*., 2022; López-Moral *et al*., 2020b), english walnut (Gusella *et al*., 2020; López-Moral *et al*., 2020a, 2023), almond (Gramaje *et al*., 2012), avocado (Guarnaccia *et al*., 2016, 2020; Hernández *et al*., 2023), mango (Aiello *et al*., 2022; Ismail *et al*., 2012, 2013; Polizzi *et al*., 2023) fig tree (Aiello *et al*., 2020; Çeliker *et al*., 2012; Güney *et al*., 2022), and many others. These fungi, as say above, can affect several hosts and not only cultivated. Ornamental and forestry crops are highly susceptible of these fungi and there are disease report on *Brachychiton* spp. (Gusella *et al*., 2021), giant

sequoia (Haenzi *et al*., 2021; Kovač *et al*., 2021), *Ficus* sp. (Al-Bedak *et al*., 2018), english oak (Barradas *et al*., 2013), sessile oak (Vajna, 1986), algerian oak (Linaldeddu *et al*., 2013), olm and cork oak (Alves *et al*., 2004) hybrid *Rhododendrons* (Pintos Varela *et al*., 2011), *Phoenix* spp. (Ligoxigakis *et al*., 2013), various coniferous (Alves *et al*., 2013), *Fraxinus* sp. (Alves *et al*., 2014), carob tree (Granata *et al*., 2011), and many others. Moreover, *Botryosphaeriaceae* are considered opportunistic because they can cause disease when the host is under stress and may lead to the host death (Slippers and Wingfield, 2007). Specifically, environmental stress factors that can contribute to infections by *Botryosphaeriaceace* species include drought, hail, frost, wind injuries, lesions caused by other pathogens or insect pests, competition with other plants for water and mineral elements, as well as plantations established in unsuitable environments (Paoletti *et al*., 2001; Zwolinski *et al*., 1990).

These fungi can attack several organs of the tree and in different physiological stages. *Botryosphaeriaceae* are important pathogens usually associated with canker symptoms, but also causing leaf spots, twig blight, dieback, internal discoloration, and general wilting. The pathogens have the ability to infect the host trough the wounds and natural opening (Yan *et al*., 2018). In the past, there was a prevailing belief that *Botryosphaeriaceae* exhibited host-specificity or had the ability to infect only a restricted range of hosts. Consequently, numerous species were described based on this assumed host-specific behavior. Meanwhile, recent studies have demonstrated that many species have a low level of host-specificity and some species, such as *Neofusicoccum parvum* are highly polyphagous (Phillips *et al*., 2013). Various factors contribute to the heightened virulence of certain *Botryosphaeriaceae* species, enabling them to transition effectively from one host to another. A recent study (Garcia *et al*., 2021) highlighted how specific taxa groups like *Botryosphaeria*, *Lasiodiplodia*, and *Neofusicoccum* exhibit an expansion of clades of

gene families, CAZymes, transporter and secondary metabolism, associated with pathogenesis.

Regarding the ecology of *Botryosphaeriaceae*, that is complex and not fully understood, is well know that they are also endophytes on many hosts often coexisting in the same tissue and forming latent infection (Slippers and Wingfield, 2007). During the infection process that fungi start the tissue colonization and remain in latent phase for long time, and when the host is subjected of some stress factor the pathogens can cause disease symptoms. This aspect must be taken into serious consideration because many pathogens can be spread throughout the young plant from the nursery to the open field. For instance, recent studies, conducted in California, help us to quantify *Botryosphaeriaceae* inoculum source in different symptomatic and asymptomatic woody host by using Real-time qPCR technique (Luo *et al*., 2017, 2019, 2020, 2021). The climate-change have also influence on pathogen, host, and pathogen-host interaction (Elad and Pertot, 2014). In particular, the change associated with global warming, such us increased temperature, change in quantity and pattern of precipitation, increase CO_2, and ozone levels, drought, hail, etc. may affect the incidence and severity of plant disease and influence the further coevolution of plants and their pathogens (Batista *et al*., 2023; Burdon *et al*., 2006; Chakraborty, 2005; Crowl *et al*., 2008; Eastburn *et al*., 2011; Garrett *et al.,* 2006). Recent study based on *Botryosphaeriaceae*-related risk assessment (Batista *et al*., 2023) showed that the role of climate-change could severely impact agriculture and forestry production. In detail an overall increase of suitable areas, a possible range expansion in norther hemisphere, and a consistent intensify in number of months with optimal conditions for these fungi were predicted (Batista *et al*., 2023). Moreover, the adverse weather events such as the alteration of wet and dry cycles, extreme high and low temperatures during the seasons can be favor pathogen diffusion and jump host (Elena *et al*., 2006; Markakis *et al*.,

2017, 2019). For those reasons the incidence of *Botryosphaeriaceae* species causing trunk disease symptoms on woody host increase during the years (Chakraborty and Newton, 2011; Guarnaccia *et al*., 2022). Nowadays, many species in this family have been reported on different crops in Sicily (Italy). Among them, several studies report traditional crop infected by *Botryosphaeriaceae* such as *Citrus* (Bezerra *et al*., 2021; Polizzi *et al*., 2009), pistachio (Gusella *et al*., 2022), grapevine (Burruano *et al*., 2008; Mondello *et al*., 2013, 2020), english walnut (Gusella *et al*., 2020), cactus pear (Aloi *et al*., 2020; Schena *et al*., 2018), loquat (Giambra *et al*., 2016) and more. Unfortunately, traditional crops are not the only threatened by these fungi. In the last few years, an increasing number of farmers invested in innovative crops in Sicily, such as tropical crops. Between of them there are avocado (*Persea americana*), mango (*Mangifera indica*), litchi (*Litchi chinensis*), passion fruit (*Passiflora edulis*) and lime (*Citrus aurantifolia*). These innovative crops mainly cultivated along the cost of the Island, cause the environmental condition close to those of Tropical Countries, are susceptible to different diseases, including *Botryosphaeriaceae*, that are reported mainly on avocado (Guarnaccia *et al*., 2016), mango (Aiello *et al*., 2022; Ismail *et al*., 2013; Polizzi *et al*., 2023) and litchi (Aiello *et al*., 2022). Furthermore, these innovative crops need investigation regarding the phytopathological status because they can share pathogens between native and non-native crops in Sicily, and for this reasons research activity were carried out on those crops under the chapter “other research activities” during these Ph.D. years. Regarding avocado, that is the main tropical crops in Sicily, it was introduced as ornamental purpose, and when the demand of tropical fruits has increased worldwide, lemon production was slowly replaced by avocado (Guarnaccia *et al*., 2016) and nowadays it represents an important economic crop for the Island, and probably it will be one of the main cultivated crops in the warm regions.

For these reasons, the propose of the first part of the present Ph.D. thesis is to investigate the diversity of *Botryosphaeriaceae* species causing disease symptoms in the main avocado orchards in Sicily, in order to characterize the recovered species from symptomatic samples, and to test their pathogenicity to this host.
At the same time, as well known, *Botryosphaeriaceae* species affect not only cultivated crops but also ornamental plants that are often under investigate and represent an important source of inoculum. Ornamental plants play a crucial role in in beautifying landscape and these infections compromise the overall health and appearance of the trees. In the last decade, there was an increase of phytopathological cases imputably to *Botryosphaeriaceae* species, for example on *Brachychiton* spp. (Gusella *et al*., 2021), as well as on ornamental fig (*Ficus carica*) (Aiello *et al*., 2020), and on Indian hawthorn (*Rhaphiolepis indica*) (Gusella *et al*., 2020). During the recent years several decline symptoms were consistently observed in Indian laurel-leaf fig (*Ficus microcarpa*) in different urban area and in *Acacia* species in nursery in Sicily. Regarding Indian laurel-leaf fig, it is considered one of the most common urban trees in warm climates worldwide (Riffle, 1998) and were introduced in Southern Italy as ornamental species. Nowadays is common in many urban areas and viewed as an important form of historical heritage (Fici and Raimondo, 1996). Regarding the nursery, is well known that *Botryosphaeriaceae* can start the infection in young plants and remain in latent phase also after the transplant in the field, and cause disease when the host is under stress. Therefore, is important investigate the propagation step, because could represent an important phase for fungal infection. For these reasons, ornamental crops need deeply studies, and the second and third part of the present Ph.D. thesis aimed to investigate the etiology of Indian laurel-leaf fig and *Acacia* spp. disease trough characterizing the fungal isolates recovered from diseased plants based on a multi-loci phylogenetic analysis and to test

their pathogenicity.
In conclusion, *Botryosphaeriaceae* are highly polyphagous fungi, can infect the host when it is under stress and are latent pathogen that easily favor the spread of asymptomatic plant from nursery to open field. For these reasons this fungal family represent a serious threat for agricultural, ornamental and forest ecosystem.

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2. Experimental part 1: Diversity of *Botryosphaeriaceae* species associated with canker and dieback on avocado (*Persea americana*) in Italy

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2.1 Introduction

Avocado (*Persea americana* L.) is a tree native to Mexico and has spread to many tropical and subtropical regions (Bost *et al*., 2013). Consumption of avocado fruit and new plantings of avocados has considerably increased (Bost *et al*., 2013). Greatest production is in Mexico, followed by Colombia and the Dominican Republic (FAOSTAT 2022). In Europe, Spain was the first country to developed commercial production of avocados (Pérez-Jiménez, 2008). In Italy, avocado production is spread in the Southern regions, mainly in Sicily, where the cultivated area has increased in the last 10 years (Migliore *et al*., 2017). In Sicily, avocado provides good agricultural diversification as an alternative crop to *Citrus* (Guarnaccia *et al*., 2016).

Several diseases can affect avocados, and several fungi taxa have been associated with different symptoms. Traditionally, root diseases have been considered the most important limiting factors for the avocado production. Among these, those caused by *Phytophthora cinnamomi* and *Rosellinia necatrix* are considered the most important and widespread diseases of avocado, leading to serious losses, especially in the Mediterranean regions where avocado production is well established (Fiorenza *et al*., 2021; López-Herrera and Melero-Vara, 1992; Zentmyer, 1980). In recent years, species of *Nectriaceae* have also been shown to be important, especially in Australia where different taxa have been associated with crown root rot disease (Parkinson *et al*., 2017). In Italy, recent studies have shown the presence of *Nectriaceae* spp. causing a complex of root symptoms (Aiello *et al*., 2020b; Vitale *et al*., 2012).

In recent decades, increased research has been carried out on canker diseases of fruit and nut crops (Guarnaccia *et al*., 2022a; Moral *et al*., 2019). These diseases have been re-discovered as important and

limiting for perennial crops, especially because they cause polyetic epidemics, a complex of pathogen taxa are involved, and most of the causal agents are polyphagous and live as latent pathogens. Among the taxa associated and responsible for shoot, branch and trunk cankers and dieback, *Botryosphaeriaceae* is a widely investigated group of fungi (Batista *et al.,* 2021). *Botryosphaeriaceae* includes fungi that can be pathogens, saprobes and endophytes (Phillips *et al*., 2013; Slippers and Wingfield, 2007), and can be severe threats to fruit, nut, ornamental and forest trees (Moral *et al*., 2019; Slippers and Wingfield, 2007). DNA-based tools, especially multi-locus phylogeny, have shown that many genera and species within the *Botryosphaeriales* have been described, synonymized, and re-accommodated (Zhang *et al*., 2021).

On avocado, despite sporadic reports of *Diaporthe* species associated with cankered tissues (Guarnaccia *et al*., 2016; Mathioudakis *et al*., 2020; Torres *et al*., 2016), different *Botryosphaeriaceae* have been extensively reported worldwide causing canker and dieback on woody tissues and fruit rots, including: *Botryosphaeria dothidea*, *Diplodia aromatica, D. dominicana, D. mutila*, *D. pseudoseriata*, *D. seriata*, *Dothiorella iberica, Lasiodiplodia laeliocattleyae*, *L. pseudotheobromae, L. theobromae*, *Neofusicoccum australe*, *N. luteum, N. mangiferae, N. mediterraneum, N. nonquaesitum*, *N. parvum*, and *N. stellenboschiana* (Arjona-Girona *et al*., 2019; Auger *et al*., 2013; Avenot *et al*., 2022; Carrillo *et al*., 2016; Dann *et al*., 2013; Guarnaccia *et al*., 2020; Hartill, 1991; Hartill and Everett, 2002; McDonald *et al*., 2009; McDonald and Eskalen, 2011; Ni *et al*., 2009; Ni *et al*., 2011; Peterson, 1978; Qiu *et al*., 2020; Rodríguez-Gálvez *et al*., 2021; Tapia *et al*., 2020; Twizeyimana *et al*., 2013; Valencia *et al*., 2019; Wanjiku *et al*., 2020; Zea-Bonilla *et al*., 2007). On avocado, canker and dieback symptoms can appear on shoots, branches, and trunks. Usually, reddish sap that became white/beige with the age has been associated with cankers. The tree bark can be friable or sunken

and necrotic, showing cracking, with external dark discolouration. Internally, infected wood becomes brown with characteristic wedge-shaped discolourations affecting the xylem. Under high disease pressure, severe xylem colonization may be observed, with associated wilting of shoots and leaves, that remain attached.

In Italy, the first investigations of avocado branch and trunk canker were reported in 2016, showing the presence of *Botryosphaeriaceae* (*N. parvum*), *Diaporthaceae* (*D. foeniculacea* and *D. sterilis*) and *Glomerellaceae* (*Colletotrichum gloeosporioides* and *C. fructicola*) (Guarnaccia *et al*., 2016). Studies on avocado canker diseases in Italy have continued, and in 2018, a new species *Neocosmospora persea* was described, causing branch and trunk canker, which was also later reported in Greece (Guarnaccia *et al*., 2018, 2022b). More recently, the new species *Neopestalotiopsis siciliana* and *Ne. rosae* were reported as causing stem lesions and dieback on avocado (Fiorenza *et al*., 2021).

An increased incidence of shoot and branch canker has been observed in Sicilian avocado orchards since 2016. The present study has investigated the diversity of *Botryosphaeriaceae* associated with symptomatic trees. The aims of the study were: (i) to characterize the *Botryosphaeriaceae* recovered from symptomatic avocado samples, and (ii) to test their pathogenicity to this host.

2.2 Materials and methods

2.2.1 Field surveys and fungal isolation

Surveys were conducted in Sicily (Italy) during 2020 and - 2021 in the main avocado production areas (Catania, Messina, and Siracusa provinces). Eleven orchards were investigated and selected for sampling. Samples (three to ten plants from each site) of symptomatic branches, trunks and shoots were collected, and brought

to the Plant Pathology laboratory, Dipartimento di Agricoltura, Alimentazione e Ambiente, Sezione di Patologia Vegetale, University of Catania. Small sections (0.5 × 0.5 cm^2) of symptomatic tissues were surface disinfected for 1 min in 1.5% sodium hypochlorite solution (NaOCl), rinsed in sterile distilled water, dried on sterile absorbent paper and placed on potato dextrose agar (PDA; Lickson) amended with 100 mg L^{-1} of streptomycin sulphate (Sigma-Aldrich) to prevent bacterial growth, and incubated at 25±1°C for 7 d. Isolation frequency of *Botryosphaeriaceae* was calculated using the formula: $F = (N_{Bot}/N_{Tot}) \times 100$, where F is the frequency of *Botryosphaeriaceae*; N_{Bot} is the number of woody fragments from which *Botryosphaeriaceae* were isolated; and N_{Tot} is the total number of woody fragments from which fungi were isolated. Single hyphal tip cultures on PDA were obtained. These isolates are maintained in the Plant Pathology collection of the University of Catania.

2.2.2 Morphological and culture characters of isolates

Representative isolates of each morphologically different group of isolates were transferred onto Technical Agar (AT, 1.2% Agar Technical, Biolife) supplemented with autoclaved pine needles (Smith *et al.*, 1996), and were incubated at room temperature under UV light. The size, colour, and shape of conidia produced by the isolates were examined. After 14 d, pycnidia were observed with a stereoscope, and were mounted in 100% lactic acid. Fifty conidia from each representative isolate were measured (length and width), using an Olympus-BX61 fluorescence microscope coupled to an Olympus DP70 digital camera. Measurements were captured using the software analySIS 3.2 (Soft Imaging System GmbH). Dimensions are reported here as averages.

2.2.3 DNA extraction and PCR analyses

The representative isolates were cultivated on PDA for 7 d, and genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation) following the manufacture's protocol. The quality of the DNA was determined using a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific), and was diluted to 5 ng μL^{-1} with nuclease-free water. The internal transcriber spacer region (ITS) of the nuclear ribosomal RNA operon was amplified with primers ITS5 and ITS4 (White *et al.*, 1990), and the primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used to amplify part of the translation elongation factor 1alpha locus (*tef1*-α), and primer sets Bt2a and Bt2b (Glass and Donaldson, 1995) were used for the partial beta tubulin locus (*tub2*). The amplifications were each carried out in a final volume of 25 µL using One Taq® 2× Master Mix with Standard Buffer (BioLabs), according to the manufacturer's instructions, on an Eppendorf Mastercycler (AG 22331). The PCR consisted of initial 30 s at 94°C, followed by 35 cycles at 94°C for 30 s, 50-52°C (ITS), 57–59°C (*tef1-α*), or 52°C (*tub2*) for 1 min, followed by 68°C for 1 min, and 5 min at 68°C. All PCR products were visualized on 1% agarose gels (90 V for 40 min), stained with GelRed®, purified, and sequenced by Macrogen Inc. Forward and reverse DNA sequences were assembled and edited using AliView software (Larsson, 2014), and were submitted to GenBank. Sixty isolates were sequenced (amplifying the *tub2* locus only), and based on these preliminary results only 23 representative isolates were considered for further locus sequencing and phylogenetic analyses.

2.2.4 Phylogeny

Sequences were read, assembled, and edited using MEGAX:

Molecular Evolutionary Genetics Analysis across computing platforms (Kumar *et al*., 2018). The ITS, *tub2* and *tef1*-α DNA sequence datasets were aligned using MEGAX. For comparison, 57 additional sequences were selected according to the most recent taxonomic classification of *Botryosphaeriaceae* genera and species involved in this study (Table 1). Two analyses were performed. Maximum parsimony analysis (MP) was performed in PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0a (Swofford, 2002). The analysis of the combined dataset (ITS + *tub2* + *tef1*-α) was obtained with the heuristic search function and tree bisection and reconstruction (TBR) as branch swapping algorithms with the branch swapping option set on 'best trees' only. Gaps were treated as 'missing', the characters were unordered and of equal weight, and Maxtrees were limited to 100. Tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated. A total of 1000 bootstrap replicates were performed to test the robustness of the tree topologies. The best-fit model of nucleotide evolution for each locus, according to the Akaike information criterion (AIC), was evaluated using MrModeltest v. 2.4 (Nylander, 2004). The Maximum Likelihood analysis (ML) of the combined loci was performed in GARLI v.0.951 (Zwickl, 2006), and clade support was assessed by 1000 bootstrap replicates. *Phyllosticta ampelicida* (CBS 111645) and *Phyllosticta citricarpa* (CBS 102374) served as the outgroup taxa in both analyses.

Table 1. Information of fungal isolates used in the phylogenetic analysis and their corresponding GenBank accession numbers. Isolates in bold are from this study. Letter "T" in apex identifies type material.

Species	Isolate ID	Host	Country	GenBank accession no.		
				ITS	*tef1*-α	*tub2*
Botryosphaeria agaves	CBS 133992 = MFLUCC 11-0125^{T}	*Agave* sp.	Thailand	JX646791	JX646856	JX646841
Botryosphaeria agaves	CBS 141505 = CPC 26299	*Agave* sp.	France	KX306750	MT592030	MT592463
Botryosphaeria corticis	CBS 119047^{T} CAP 197	*Vaccinium corymbosum*	New Jersey, USA	DQ299245	EU017539	EU673107
Botryosphaeria corticis	CBS 119048 = CAP 198	*Vaccinium corymbosum*	New Jersey, USA	DQ299246	EU017540	MT592464
Botryosphaeria dothidea	CBS 115476 = CMW 8000^{T}	*Prunus* sp.	Switzerland	AY236949	AY236898	AY236927
Botryosphaeria dothidea	CBS 110302 = CAP 007	*Vitis vinifera*	Portugal	AY259092	AY573218	EU673106
Botryosphaeria dothidea	**AB2**	***Persea americana***	**Catania, Sicily, Italy**	**OP654490**	**OP764459**	**OP764436**
Botryosphaeria dothidea	**AB4**	***Persea americana***	**Catania, Sicily, Italy**	**OP654491**	**OP764460**	**OP764437**
Botryosphaeria dothidea	**AB5**	***Persea americana***	**Catania, Sicily, Italy**	**OP654492**	**OP764461**	**OP764438**
Botryosphaeria dothidea	**AC5**	***Persea americana***	**Catania, Sicily, Italy**	**OP654493**	**OP764462**	**OP764439**
Botryosphaeria dothidea	**AC7**	***Persea americana***	**Catania, Sicily, Italy**	**OP654494**	**OP764463**	**OP764440**
Botryosphaeria dothidea	**AC9**	***Persea americana***	**Catania, Sicily, Italy**	**OP654495**	**OP764464**	**OP764441**
Botryosphaeria dothidea	**AC10**	***Persea americana***	**Catania, Sicily, Italy**	**OP654496**	**OP764465**	**OP764442**

Botryosphaeria dothidea	**AC11**	***Persea americana***	**Catania, Sicily, Italy**	**OP654497**	**OP764466**	**OP764443**
Botryosphaeria fabicerciana	CBS 118831 = CMW 14009	*Syzygium cordatum*	South Africa	DQ316084	MT592032	MT592468
Botryosphaeria fabicerciana	CBS 127193 = CMW 27094^{T}	*Eucalyptus* sp.	China	HQ332197	HQ332213	KF779068
Botryosphaeria kuwatsukai	CGMCC 3.18007^{T}	*Malus sp.*	China	KX197074	KX197094	KX197101
Botryosphaeria kuwatsukai	CGMCC 3.18008	*Amygdalus* sp.	China	KX197075	KX197095	KX197102
Botryosphaeria qingyuanensis	CERC 2946 = CGMCC 3.18742^{T}	*Eucalyptus* hybrid	China	KX278000	KX278105	KX278209
Botryosphaeria qingyuanensis	CERC 2947 = CGMCC 3.18743	*Eucalyptus* hybrid	China	KX278001	KX278106	KX278210
Botryosphaeria ramosa	CERC 2001 = CGMCC 3.187396	*Eucalyptus* hybrid	China	KX277989	KX278094	KX278198
Botryosphaeria ramosa	CBS 122069 = CMW 26167^{T}	*Eucalyptus camaldulensis*	Australia	EU144055	EU144070	KF766132
Lasiodiplodia citricola	CBS 124706 = IRAN 1521C	*Citrus* sp.	Iran	GU945353	GU945339	KU887504
Lasiodiplodia citricola	CBS 124707 = IRAN 1522C^{T}	*Citrus* sp.	Iran	GU945354	GU945340	KU887505
Lasiodiplodia citricola	CGMCC 3.19022	*Vaccinium corymbosum*	China	MH330318	MH330327	MH330324
Lasiodiplodia citricola	**AC20**	***Persea americana***	**Catania, Sicily, Italy**	**OP654498**	**OP764481**	**OP764444**
Lasiodiplodia euphorbiaceicola	CMM 3609^{T}	*Jatropha curcas*	Brazil	KF234543	KF226689	KF254926
Lasiodiplodia euphorbiaceicola	CMW 33268	*Adansonia* sp.	Senegal	KU887131	KU887008	KU887430
Lasiodiplodia mahajangana	CBS 124925 = CMW 27801^{T}	*Terminalia catappa*	Madagascar	FJ900595	FJ900641	FJ900630
Lasiodiplodia mahajangana	CBS 124926 = CMW 27818	*Terminalia catappa*	Madagascar	FJ900596	FJ900642	FJ900631

Lasiodiplodia mediterranea	CBS 137783 = BL 1[T]	*Quercus ilex*	Italy	KJ638312	KJ638331	KU887521
Lasiodiplodia mediterranea	CBS 137784 = BL 101	*Vitis vinifera*	Italy	KJ638311	KJ638330	KU887522
Lasiodiplodia parva	CBS 456.78[T]	*Cassava*	Colombia	EF622083	EF622063	KU887523
Lasiodiplodia parva	CBS 494.78	*Cassava*	Colombia	EF622084	EF622064	EU673114
Lasiodiplodia viticola	CBS 128313 = UCD 2553AR[T]	*Vitis vinifera*	USA	HQ288227	HQ288269	HQ288306
Lasiodiplodia viticola	CBS 128314 = UCD 2604MO	*Vitis vinifera*	USA	HQ288228	HQ288270	HQ288307
Macrophomina euphorbiicola	CMM4134 / CCMF-CNPA 288[T]	*Ricinus communis*	Brazil	KU058936	KU058906	MF457658
Macrophomina euphorbiicola	CMM4145 / CCMF-CNPA 289	*Ricinus communis*	Brazil	KU058937	KU058907	MF457659
Macrophomina phaseolina	CBS 227.33[T]	*Zea mays*		KF531825	KF531804	KF531806
Macrophomina phaseolina	KARE1339	*Pistacia vera*	USA	MN097202	MN106057	MN106087
Macrophomina phaseolina	**AC28**	***Persea americana***	**Catania, Sicily, Italy**	**OP654499**	**OP764467**	**OP764445**
Macrophomina phaseolina	**AC29**	***Persea americana***	**Catania, Sicily, Italy**	**OP654500**	**OP764468**	**OP764446**
Macrophomina phaseolina	**AC51**	***Persea americana***	**Catania, Sicily, Italy**	**OP654501**	**OP764469**	**OP764447**
Macrophomina phaseolina	**AC52**	***Persea americana***	**Catania, Sicily, Italy**	**OP654502**	**OP764470**	**OP764448**
Macrophomina pseudophaseolina	CPC 21417	*Arachis hypogaea*	Senegal	KF951791	KF952153	KF952233
Macrophomina pseudophaseolina	CPC 21524	*Hibiscus sabdarifa*	Senegal	KF951799	KF952161	KF952240
Macrophomina tecta	BRIP 70781[T]	*S. bicolor*	Chinchilla, Qld	MW591684	MW592271	MW592300

Macrophomina tecta	BRIP 71603	*S. bicolor*	Chinchilla, Qld	MW591631	MW592218	MW592301
Macrophomina vaccinii	CGMCC 3.19503^{T}	*V. corymbosum* × *V. darrowii*	China	MK687450	MK687426	MK687434
Macrophomina vaccinii	CGMCC 3.19504	*V. corymbosum* × *V. darrowii*	China	MK687451	MK687427	MK687435
Neofusicoccum australe	CBS 139662 = CMW 6837^{T}	*Acacia* sp.	Australia: Victoria	AY339262	AY339270	AY339254
Neofusicoccum australe	CBS 113220 = CMW 6853	*Sequoiadendron*	Australia	AY339263	AY339271	AY339255
Neofusicoccum cryptoaustrale	CBS 122813 = CMW 23785^{T}	*Eucalyptus* sp.	South Africa	FJ752742	FJ752713	FJ752756
Neofusicoccum cryptoaustrale	**AVORAM1**	***Persea americana***	**Catania, Sicily, Italy**	**OP654508**	**OP764476**	**OP764454**
Neofusicoccum cryptoaustrale	**AVORAM2**	***Persea americana***	**Catania, Sicily, Italy**	**OP654509**	**OP764477**	**OP764455**
Neofusicoccum cryptoaustrale	**AVORAM3**	***Persea americana***	**Catania, Sicily, Italy**	**OP654510**	**OP764478**	**OP764456**
Neofusicoccum cryptoaustrale	**AVORAM4**	***Persea americana***	**Catania, Sicily, Italy**	**OP654511**	**OP764479**	**OP764457**
Neofusicoccum cryptoaustrale	**AVORAM5**	***Persea americana***	**Catania, Sicily, Italy**	**OP654512**	**OP764480**	**OP764458**
Neofusicoccum lumnitzerae	CBS 139674 = CMW 41469^{T}	*Lumnitzera racemosa*	South Africa	KP860881	KP860724	KP860801
Neofusicoccum lumnitzerae	CBS 139675 = CMW 41228	*Lumnitzera racemosa*	South Africa	MT587480	MT592193	MT592685
Neofusicoccum luteum	CBS 110497 = CPC 4594 = CAP 037	*Vitis vinifera*	Portugal	EU673311	EU673277	EU673092
Neofusicoccum luteum	CBS 110299 = LM 926 = CAP 002^{T}	*Vitis vinifera*	Portugal	AY259091	KX464688	DQ458848
Neofusicoccum luteum	**AVF3**	***Persea americana***	**Catania, Sicily, Italy**	**OP654503**	**OP764471**	**OP764449**
Neofusicoccum luteum	**AVF5**	***Persea americana***	**Catania, Sicily, Italy**	**OP654504**	**OP764472**	**OP764450**

Neofusicoccum luteum	**AVF6**	***Persea americana***	**Catania, Sicily, Italy**	**OP654505**	**OP764473**	**OP764451**
Neofusicoccum luteum	**AVF7**	***Persea americana***	**Catania, Sicily, Italy**	**OP654506**	**OP764474**	**OP764452**
Neofusicoccum luteum	**AVF8**	***Persea americana***	**Catania, Sicily, Italy**	**OP654507**	**OP764475**	**OP764453**
Neofusicoccum mediterraneum	CBS 121558	*Olea europea*	Italy	GU799463	GU799462	GU799461
Neofusicoccum mediterraneum	CBS 121718 = CPC 13137[T]	*Eucalyptus* sp.	Greece	GU251176	GU251308	GU251836
Neofusicoccum protearum	CBS 114176 = CPC 1775 = JT 189[T]	*Leucadendron salignum × L. laureolum*	South Africa	AF452539	KX464720	KX465006
Neofusicoccum protearum	CBS 115177 = CPC 4357	*Protea magnifica*	South Africa	FJ150703	MT592239	MT592731
Neofusicoccum stellenboschiana	CBS 110864 = CPC 4598	*Vitis vinifera*	South Africa	AY343407	AY343348	KX465047
Neofusicoccum terminaliae	CBS 125263 = CMW 26679[T]	*Terminalia sericea*	South Africa	GQ471802	GQ471780	KX465052
Neofusicoccum terminaliae	CBS 125264 = CMW 26683	*Terminalia sericea*	South Africa	GQ471804	GQ471782	KX465053
Neofusicoccum ursorum	CBS 122811 = CMW 24480[T]	*Eucalyptus* sp.	South Africa	FJ752746	FJ752709	KX465056
Neofusicoccum ursorum	CBS 122812 = CMW 23790	*Eucalyptus* sp.	South Africa	FJ752745	FJ752708	KX465057
Neofusicoccum viticlavatum	CBS 112878 = CPC 5044 = JM 86[T]	*Vitis vinifera*	South Africa	AY343381	AY343342	KX465058
Neofusicoccum viticlavatum	CBS 112977 = STE-U 5041	*Vitis vinifera*	South Africa	AY343380	AY343341	KX465059
Phyllosticta ampelicida	CBS 111645	*Taxus baccata*	Nethherlands	FJ824766	FJ824773	FJ824779
Phyllosticta citricarpa	CBS 102374	*Citrus aurantium*	Brazil	FJ824767	FJ538371	FJ824778

2.2.5 Pathogenicity test

A pathogenicity test was conducted in a greenhouse, February to April 2022. Five 2-year-old asymptomatic avocado plants *'Hass'* grafted on *'Zutano'* rootstocks were selected for each tested fungal species. Inoculations were each carried out using a mycelium plug (0.5 cm^2) from a 10-d-old culture of each of *Botryosphaeria dothidea* (AC7), *Lasiodiplodia citricola* (AC20), *Macrophomina phaseolina* (AC29), *Neofusicoccum cryptoaustrale* (AVORAM4), and *Neofusicoccum lutem* (AVF5). Each inoculation site was first surface disinfected with a 70% ethanol solution. Two points of inoculation for each plant were made on the stem after removing a piece of bark with a sterile scalpel blade, placing the isolate mycelium plug onto the wound and covering it with Parafilm® (American National Can) to prevent desiccation. Three 2-year-old asymptomatic avocado plants were inoculated with sterile PDA plugs to serve as inoculation controls. The plants were moved to a greenhouse and regularly watered. Temperature in the greenhouse ranged from 18 to 27 °C and humidity from 70 to 80%. The inoculated plants were monitored weekly for symptom development, and a final assessment was conducted 63 d after the inoculations. Lesion length measurements were recorded, and were statistically analyzed in Statistix 10 (Analytical Software, 2013) using analysis of variance (ANOVA). Mean differences were compared with the Fisher's protected least significant difference (LSD) test at $\alpha = 0.05$. To fulfill Koch's postulates, re-isolations were carried out following the procedure described above, and each re-isolated fungus was identified through observation of morphological characteristics.

2.3 Results

2.3.1 Field surveys and fungal isolations

Disease was observed on 2 to 10-year-old avocado plants 'Hass', grafted on different rootstock cultivars ('Zutano', 'Duke 7', and 'Dusa') in Sicily (Italy). All the sampled plants showed symptoms of shoot and branch canker, and dieback emerging within the green canopies (Figure 1 A to D). Occasionally, a white powder was present on the surfaces of the lesions (Figure 1 E). It was also possible to observe the infections starting from pruning wounds (Figure 1 F and G). The bark of cankered shoots was cracked, darkly discoloured, and/or slightly sunken (Figure 1 H). Cankers were reddish-brown under the bark, and variable in shape. Necrotic lesions and internal discolouration were observed at the grafting points of young plants (Figure 1 I). Isolations frequently (41%) yielded *Botryosphaeriaceae*-like fungi, and *Botryosphaeriaceae* were detected in all the samples analyzed.

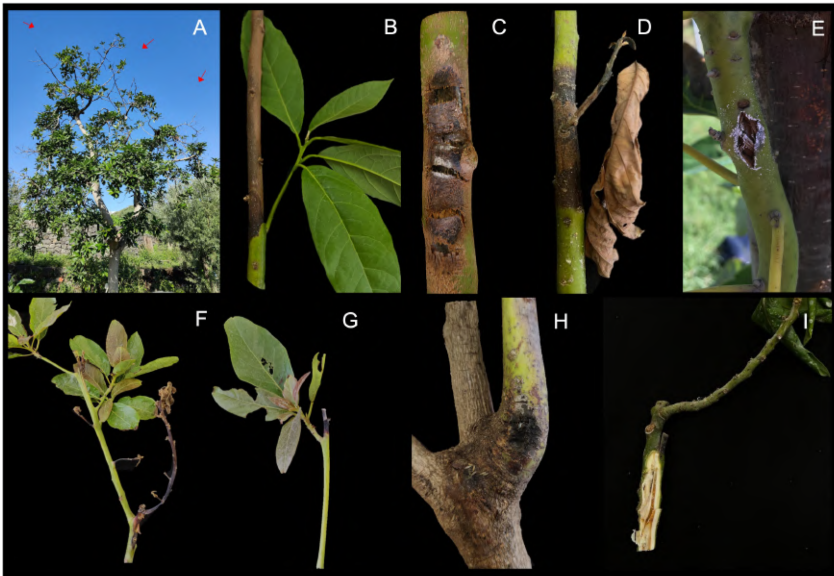


Figure 1 Symptoms of *Botryosphaeriaceae* on avocado trees observed in the field. A, Shoot dieback in the host canopy. B, Branch dieback. C and D, External canker (shoot canker). E, Canker with white powdery exudation. F and G, Infection originating from pruned wounds. H, bark cracking. I, infected grafting point.

A total of 106 *Botryosphaeriaceae* isolates were collected and stored. Of these, 62 isolates (59 %) were processed for DNA extraction, PCR, and sequencing. A preliminary screening based on the *tub2* locus was conducted on all 62 isolates, and this showed that representative isolates from seven orchards (orchard numbers 5 to 11, Table 2) were *N. parvum*. Since this fungal species was already characterized and reported in a preliminary study (Guarnaccia *et al*., 2016), these isolates were excluded from further locus sequencing and phylogenetic analyses, but the *N. parvum* isolates were collected and stored, since the present investigation showed that this fungus predominated in Sicilian avocado orchards. A total of 23 isolates derived from four orchards (orchards numbers 1 to 4) were fully characterized, as these isolates were previously unreported from in Italy. These 23 isolates were from 75 young (2 to 4-year-old) plants showing typical symptoms of canker and dieback. More details of the collected and characterized isolates are summarized in Table 2.

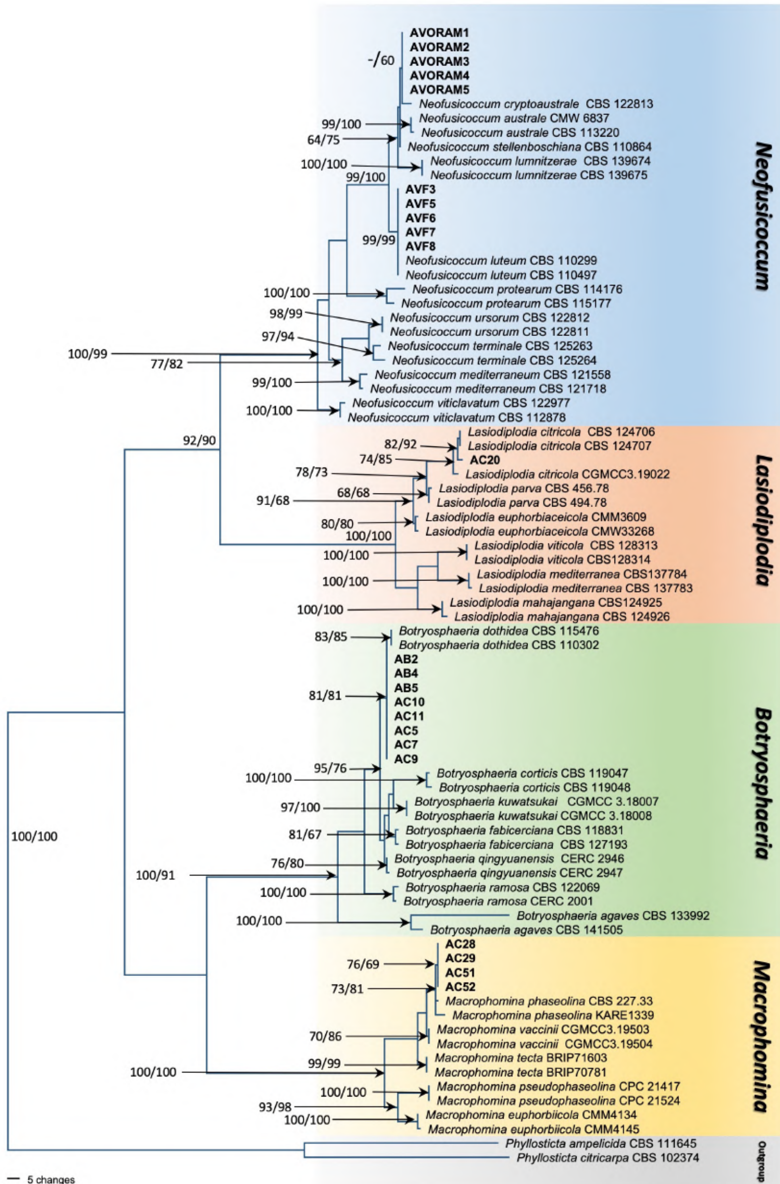
Table 2. Information on fungal isolates collected and processed in this study, from 11 avocado orchards. * identifies the representative isolates preliminarily identified based on the *tub2* locus. ¥ identifies the isolates fully characterized (ITS + *tub2*+ *tef1-α*) and included in the phylogenetic analyses. × indicates that the representative isolates were excluded from the phylogenetic analyses, because they were identified as *Neofusicoccum parvum* in the preliminary *tub2* locus characterization.

Orchard n°	Location	Tree age	Symptoms	Collected *Botryosphaeriaceae*	Number of representative isolates*	Number of chosen isolates¥	Species
1	Fiumefreddo (CT)	-	Dieback	9	8	5	*N. luteum* (5), *N. parvum* (3)
2	Ramacca (CT)	2-3 yrs	Dieback	5	5	5	*N. cryptoaustrale* (5)
3	Riposto (CT)	2-4 yrs.	Canker	5	5	5	*B. dothidea* (5)
4	Mascali (CT)	2-3 yrs.	Canker, dieback, grafting point canker	26	10	8	*B. dothidea* (3), *L. citricola* (1), *M. phaseolina* (4), *N. parvum* (2)
5	Agnone Bagni (SR)	2-4 yrs.	Canker, dieback, grafting point canker	8	8	×	*N. parvum* (8)
6	Fiandaca (CT)	-	Canker, dieback	5	2	×	*N. parvum* (2)
7	Acireale (CT)	-	Dieback, grafting point canker	7	4	×	*N. parvum* (4)
8	Messina (ME)	-	Discolouration	3	2	×	*N. parvum* (2)
9	Noto (SR)	2 yrs.	Canker, dieback, grafting point canker	17	9	×	*N. parvum* (9)
10	Giarre (CT)	Mature trees	Canker, dieback	9	1	×	*N. parvum* (1)
11	Riposto (CT)	3 yrs.	Canker	12	8	×	*N. parvum* (8)

2.3.2 Phylogeny

The MP analysis of the combined dataset showed that of 3,116 characters, 464 were parsimony-informative, 239 were parsimony-uninformative, and 2,413 were constant. A total of 100 trees were retained. Tree length was equal to 1,178, CI = 0.771, RI = 0.953, and RC = 0.735. The best-fit model of nucleotide evolution based on the AIC resulted GTR + I + G for ITS, HKY + G for *tub2* and GTR + G for *tef1*-α. The ML analysis showed that of 3,116 total characters, 2,413 were constant characters and 564 were parsimony informative. Isolates AB2, AB4, AB5, AC5, AC7, AC9, AC10 and AC11 strongly clustered within the clade of *B. dothidea* (81% MP bootstrap support and 81% ML bootstrap support). Isolate AC20 strongly grouped within the clade of *Lasiodiplodia citricola,* (74/85). Isolates AC28, AC29, AC51 and AC52 grouped in the clade of *Macrophomina phaseolina* (73/81). Regarding *Neofusicoccum*, for isolates AVORAM1 to 5 the bootstrap support was 52 for the MP analysis and 60 for the ML analysis. These isolates were accommodated within *Neofusicoccum cryptoaustrale*. Isolates AVF3, AVF5, AVF6, AVF7, AVF8 were strongly supported (99/99) within the clade of *Neofusicoccum luteum*.

According to these results, five species isolated from avocado in this study were identified, including: *B. dothidea*, *L. citricola*, *M. phaseolina*, *N. cryptoaustrale*, and *N. luteum* (Figure 2). The ITS, *tub2*, and *tef1*-α sequences generated in this study were deposited in GenBank (Table 1).



AVORAM1
AVORAM2
AVORAM3
AVORAM4
AVORAM5
-/60
Neofusicoccum cryptoaustrale CBS 122813
Neofusicoccum australe CMW 6837
Neofusicoccum australe CBS 113220
Neofusicoccum stellenboschiana CBS 110864
Neofusicoccum lumnitzerae CBS 139674
Neofusicoccum lumnitzerae CBS 139675
AVF3
AVF5
AVF6
AVF7
AVF8
Neofusicoccum luteum CBS 110299
Neofusicoccum luteum CBS 110497
Neofusicoccum protearum CBS 114176
Neofusicoccum protearum CBS 115177
Neofusicoccum ursorum CBS 122812
Neofusicoccum ursorum CBS 122811
Neofusicoccum terminale CBS 125263
Neofusicoccum terminale CBS 125264
Neofusicoccum mediterraneum CBS 121558
Neofusicoccum mediterraneum CBS 121718
Neofusicoccum viticlavatum CBS 122977
Neofusicoccum viticlavatum CBS 112878
Lasiodiplodia citricola CBS 124706
Lasiodiplodia citricola CBS 124707
AC20
Lasiodiplodia citricola CGMCC3.19022
Lasiodiplodia parva CBS 456.78
Lasiodiplodia parva CBS 494.78
Lasiodiplodia euphorbiaceicola CMM3609
Lasiodiplodia euphorbiaceicola CMW33268
Lasiodiplodia viticola CBS 128313
Lasiodiplodia viticola CBS128314
Lasiodiplodia mediterranea CBS137784
Lasiodiplodia mediterranea CBS 137783
Lasiodiplodia mahajangana CBS124925
Lasiodiplodia mahajangana CBS 124926
Botryosphaeria dothidea CBS 115476
Botryosphaeria dothidea CBS 110302
AB2
AB4
AB5
AC10
AC11
AC5
AC7
AC9
Botryosphaeria corticis CBS 119047
Botryosphaeria corticis CBS 119048
Botryosphaeria kuwatsukai CGMCC 3.18007
Botryosphaeria kuwatsukai CGMCC 3.18008
Botryosphaeria fabicerciana CBS 118831
Botryosphaeria fabicerciana CBS 127193
Botryosphaeria qingyuanensis CERC 2946
Botryosphaeria qingyuanensis CERC 2947
Botryosphaeria ramosa CBS 122069
Botryosphaeria ramosa CERC 2001
Botryosphaeria agaves CBS 133992
Botryosphaeria agaves CBS 141505
AC28
AC29
AC51
AC52
Macrophomina phaseolina CBS 227.33
Macrophomina phaseolina KARE1339
Macrophomina vaccinii CGMCC3.19503
Macrophomina vaccinii CGMCC3.19504
Macrophomina tecta BRIP71603
Macrophomina tecta BRIP70781
Macrophomina pseudophaseolina CPC 21417
Macrophomina pseudophaseolina CPC 21524
Macrophomina euphorbiicola CMM4134
Macrophomina euphorbiicola CMM4145
Phyllosticta ampelicida CBS 111645
Phyllosticta citricarpa CBS 102374
99/100
64/75
100/100
99/100
99/99
100/100
98/99
97/94
100/99
77/82
99/100
100/100
92/90
82/92
74/85
78/73
68/68
91/68
80/80
100/100
100/100
100/100
100/100
83/85
81/81
95/76
100/100
97/100
81/67
76/80
100/91
100/100
100/100
100/100
76/69
73/81
70/86
99/99
100/100
100/100
93/98
100/100
Neofusicoccum
Lasiodiplodia
Botryosphaeria
Macrophomina
Outgroup
5 changes

Figure 2 One of 100 equally most parsimonious trees generated from maximum parsimony analysis of three-loci (ITS + *tub2* + *tef1*-α) combined dataset of *Botryosphaeriaceae* species. Numbers before after slashes represent, respectively, parsimony and likelihood bootstrap values from 1,000 replicates. *Phyllosticta ampelicida* (CBS 111645) and *Phyllosticta citricarpa* (CBS 102374) were the outgroup taxa in both analyses. Isolates in bold font were generated in the present study. Bars indicate the numbers of nucleotide changes.

2.3.3 Morphological and cultural characteristic of the isolates

Observing pure cultures on PDA, a total of six groups of *Botryosphaeriaceae*-like fungi were observed:

Isolate AC5 (*B. dothidea*) had olivaceous colonies that became grey with black reverse sides. Conidia were hyaline, fusiform and measured 23.2 × 5.6 µm.

Lasiodiplodia citricola AC20 had colonies with abundant aerial mycelium that became smoke grey to olivaceous-grey or iron-grey on the surfaces and greenish grey to dark slate blue on the reverse sides. Conidia were initially hyaline, aseptate, ellipsoid to ovoid and becoming pigmented, verrucose and ovoid, and measured 21.3 × 13.1 µm.

Macrophomina phaseolina isolate AC29 had grayish fluffy aerial mycelium on the colony surfaces, which were purplish grey on the reverse sides. Abundant microsclerotia were produced on pine needles in AT medium. Conidia were 25.0 × 10.5 µm.

The colonies of isolate AVORAM4 (*N. cryptoaustrale*) were initially white with fluffy aerial mycelium, changing to straw-yellow after 3 d incubation and then to pale olivaceous-grey. Conidia were hyaline, smooth with granular contents, aseptate, fusiform, and measured 20.0 × 6.0 µm.

Isolate AVF5 (*N. luteum*) was initially white with fluffy aerial mycelium and changed to yellow after 3-4 d incubation, after which

the colour changed to pale olivaceous-grey from the middle of the colonies to the irregular margins. Conidia were hyaline, thin walled, aseptate, smooth, ellipsoidal, and measured 19.5 × 5.5 µm.
Isolates of *N. parvum* had white fluffy aerial mycelium that became grey and then black with the age. Conidia were hyaline, non-septate, and subglobose, with obtuse apices, and measured 18.2 × 6.1 µm.

2.3.4 Pathogenicity test

The pathogenicity showed that all the *Botryosphaeriaceae* species in this study were pathogenic to avocado plants, and produced similar symptoms to those observed in the field. All the inoculated species produced external and internal discolouration lesions. The inoculation controls did not show any symptoms (Figure 3).

Figure 3 Result of the pathogenicity test after 63 days. A-B, Control. C-D, Shoots inoculated with *Botryosphaeria dothidea*. E-F, *Neofusicoc- cum luteum*. G-H, *Neofusicoccum cryptoaustrale*. I-J, *Lasiodiplodia citricola*. K-L, *Macrophomina phaseolina*. Scale bar: 2 cm

After 15 d, all the inoculated trees showed dark discolouration of the outer layers of bark. In detail, *N. luteum* isolate AVF5 produced the longest lesions (mean = 55.0 mm), followed by *N. cryptoaustrale* isolate AVORAM4 (50.9 mm), *M. phaseolina* isolate AC29 (43.6 mm), *B. dothidea* isolate AC7 (37.8 mm) and *L. citricola* isolate AC20 (31.4 mm). All the inoculated fungi produced lesion lengths that were statistically different from the controls ($P < 0.05$), and only lesions from *Neofusicoccum* sp. were significantly different compared to those from *L. citricola* (Figure 4). Re-isolations showed gave colonies with the morphological characteristics the same as the inoculated species, fulfilling Koch's postulates.

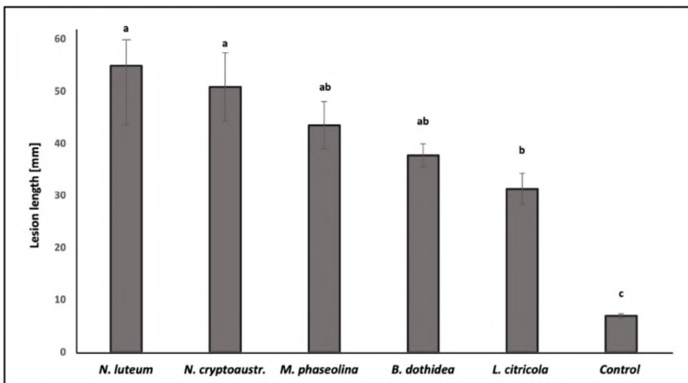


Figure 4 Mean lesion lengths (mm) resulting from the pathogenic- ity test of *Botryosphaeria dothidea, Lasiodiplodia citricola, Mac- rophomina phaseolina, Neofusicoccum cryptoaustrale,* and *N. luteum* on potted plants. Values are each for two inoculation points per plant for each fungal species. Control consisted of the same number of inoculation points. Vertical bars represent standard errors of the means. Bars accompanied with different letters indicate means that were significantly different (Fisher's protected LSD test; α = 0.05).

2.4 Discussion

This study has elucidated the diversity of *Botryosphaeriaceae* species causing avocado canker and dieback in commercial orchards in Italy. The species characterized were *B. dothidea, L. citricola, M. phaseolina, N. cryptoaustrale,* and *N. luteum*. *Neofusicoccum parvum* was also constantly encountered during field surveys. This species had been characterized in a previous study (Guarnaccia *et al*. 2016), and was here characterized only on the basis of *tub2* locus, and excluded from the phylogenetic analysis. This research confirms that *N. parvum* was the predominant Botryosphaeriaceous species associated with canker and dieback symptoms of avocado in Sicilian orchards.
Botryosphaeria dothidea is the type species of *Botryosphaeria* (Marsberg *et al*., 2017), and has been reported from many plant species with broad global distribution. There are 1,260 fungus-host records for *B. dothidea* and its synonyms listed in the Fungal Database (Farr and Rossman, 2022). However, some of these reports are outdated causing taxonomic confusion. Batista *et al*. (2021) report that *B. dothidea* was associated with 403 hosts in 66 countries. McDonald and Eskalen (2011) reported fungi belonging to the *Botryosphaeriaceae*, including *B. dothidea* (*Fusicoccum aesculi*), have been associated with avocado branch cankers in California. Previous field surveys conducted in Sicily on different perennial crops, including pistachio, walnut and *Ficus* spp., recorded presence of *B. dothidea* and other *Botryosphaeriaceae* (Fiorenza *et al*., 2022a; Gusella *et al*., 2020, 2022;). In the present study, within *Lasiodiplodia,* the *L. citricola* was occasionally isolated from symptomatic avocado branches, as were *M. phaseolina* and *B. dothidea*. The pathogenicity test confirmed the aggressiveness of *L. citricola* on avocado woody tissues. Different species of *Lasiodiplodia,* including *L. citricola*, have been reported to cause diseases in multiple fruit and nut tree hosts

(Carlucci *et al*., 2015; Chen *et al*., 2013a, 2013b, 2013c, 2014; Rodríguez-Gálvez *et al*., 2017; Úrbez-Torres *et al*., 2008, 2010). In Sicily, *L. citricola* was recently identified as a serious threat to *Acacia* spp. causing dieback (Costanzo *et al*., 2022). On avocado, recent studies have described *L. laeliocattleyae, L. pseudotheobromae* and *L. theobromae* as etiological agents of fruit stem-end rot and dieback (Avenot *et al*., 2022; Garibaldi *et al*., 2012; Qui *et al*., 2020; Rodríguez-Gálvez *et al*, 2021).

Macrophomina phaseolina is widely distributed and is a serious threat to different crops (Baird *et al*., 2003; Sarr *et al*., 2014). This pathogen causes charcoal rot of soybean (Sarr *et al*., 2014), chickpea (Dell'Olmo *et al*., 2022), sunflower (Bokor, 2007), sorghum (Sharma *et al*., 2014), and strawberry (Koike, 2008). It has also been reported to cause diseases on woody hosts, such as grapevine (González and Tello, 2011; Nouri *et al*., 2018), olive (Sergeeva *et al*., 2005), pistachio (Nouri *et al*., 2020), and almond (Inderbitzin *et al*., 2010). *Macrophomina phaseolina* was thought to be one of the pathogens causing avocado root rot in Australia (Poudel *et al*., 2021), but it has not been recorded as causing canker on this host. Based on previous studies in Italy, on fruit and ornamental hosts showing typical symptoms of *Botryosphaeriaceae*, including canker and dieback of woody tissues, *M. phaseolina* has not been previously isolated. This is the first report of *M. phaseolina* on avocado. Further investigations are required need to clarify the geographic extent this species in Italy, and its association with different host plants.

Neofusicoccum cryptoaustrale was detected in only one of the sampled avocado orchards. This fungus was first described Eucalyptus trees in South Africa (Crous *et al*., 2013; Pavlic-Zupanc *et al*., 2017), and was reported on ornamental and fruit crops, including *Pistacia lentiscus* (Linaldeddu *et al*., 2016), *Olea europea* (Hernández-Rodríguez *et al*., 2022; van Dyk *et al*., 2021), and mangrove species (Osorio *et al*., 2017). This fungus formed a cryptic sister species with

N. australe (Crous *et al*., 2013). Results of the present study showed that the isolates from avocado clustered with the type isolate of *N. cryptoaustrale* (CBS 122813), close to the well supported clade of *N. australe*. We do not exclude that the present study isolates identified as *N. cryptoaustrale* could be re-accommodated following progress with multi-locus phylogeny. *Neofusicoccum luteum* is well known as a canker pathogen of avocado, and has been reported to cause branch canker and stem-end rot on avocado in California (Avenot *et al*., 2022; McDonald *et al*., 2009; 2011; Twizeyimana *et al*., 2013;), Australia (Tan *et al*., 2019), New Zealand (Hartill, 1991; Hartill and Everett 2002), and Chile (Tapia *et al*., 2020). This fungus was also identified in California as the main cause of stem-end rot in harvested avocado fruit (Twizeyimana *et al*., 2013).
Despite of the diversity of *Botryosphaeriaceae* identified in the present study, *N. parvum* was the most prevalent species associated with canker and dieback of avocado, since it was detected from seven sampled locations with a high isolation frequency, as was previously reported in Italy by Guarnaccia *et al*. (2016) and in Spain by Arjona-Girona *et al*. (2019). *Neopestalotiopsis* also came from symptomatic tissues showing cankers and discolouration, but was not included in this study since it was already reported and described by Fiorenza *et al*. (2022b). Pathogenicity tests showed that representative isolates caused lesions on healthy plants. These data demonstrated that all the inoculated fungi were pathogenic to avocado, and that the isolates characterized as *N. cryptoaustrale* and *N. luteum* were the most virulent compared those of *B. dothidea, M. phaseolina*, and *L. citricola*.
Botryosphaeriaceae species have been reported as pathogens of the ornamentals to the agricultural crops in Italy, especially in Sicily (Aiello *et al*., 2020a, 2022; Bezerra *et al*., 2021; Costanzo *et al*., 2022; Fiorenza *et al*., 2022a; Guarnaccia *et al*., 2016; Gusella *et al*., 2020, 2021, 2022; Ismail *et al*., 2013). Of the studies in Italy,

Botryosphaeriaceae have been commonly encountered in different hosts and environments. Presence of contiguous susceptible hosts and the polyphagous behaviour of this pathogen family can guarantee inoculum survival in nurseries, open fields, and urban areas. The fungi characterized in the present study have also been described on other hosts. *Botryosphaeriaceae* (including those detected in this study) are endophytes, able to induce latent infections (Slippers and Wingfield, 2007). It is possible to detect the levels of latent infections using qPCR (Luo *et al*., 2017, 2019, 2020, 2021).

The orchards investigated in the present study contained mainly young avocado trees (2 to 4-year-old). Presence of *Botryosphaeriaceae* spp. within the tissues in young trees indicates that most of the infections may originate nurseries, and then spread once the trees are transplanted in open fields. In Sicily, avocado trees are imported from other Mediterranean countries, because there are no nurseries specialized in avocado propagation. For these reasons, monitoring of latent infections, and attention during nursery propagation, are needed to avoid or limit *Botryosphaeriaceae* infections and new sources of inoculum.

This study presents updated results on the association of *Botryosphaeriaceae* species causing canker and dieback on avocado in Italy. The surveys and analyses have elucidated the diversity of this group of fungi involved in avocado canker diseases. Further studies are required to elucidate the epidemiology, control, and latent pathogenic status of *Botryosphaeriaceae* on avocado. This study is also the first to report *L. citricola*, *M. phaseolina* and *N. cryptoaustrale* causing canker and dieback on avocado trees, and is the first report of the recorded fungi causing branch disease on avocado in Italy.

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3. Experimental part 2: A New Disease for Europe of *Ficus microcarpa* Caused by *Botryosphaeriaceae* Species

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3.1 Introduction

Ficus microcarpa, commonly known as Chinese or Malayan banyan, Indian laurel-leaf fig, and curtain fig, is a widely distributed evergreen ornamental species belonging to the family *Moraceae*, native to Ceylon, India, southern China, the Ryukyu Islands, Australia, and New Caledonia (Wagner *et al*., 1999). It is considered one of the most common urban trees in warm climates worldwide (Riffle, 1998). Moreover, *F. microcarpa* is also well known as an invader species due to its ability to grow in inhospitable places, its large fruit production, and its numerous dispersal agents (birds, bats, rodents, and others) (Starr *et al*., 2003). Many *Ficus* spp. were introduced in southern Italy as ornamental species; they are now common in many urban areas and viewed as an important form of historical heritage (Fici *et al*., 1996). Parks and gardens in urban areas are of significant value for all people living their daily lives in the cities. Urban trees have a positive impact on reducing heat, providing a convenient shelter, reducing wind velocity, and increasing the aesthetic value of the landscape (Heisler, 1977; Scott *et al*., 1999; Tyznik, 1981). In addition, most people living in cities deal with schedules, work, appointments, meetings, etc., and urban parks and open spaces positively affect mental health (Wolf, 1998). Thus, it is important not to underestimate the health of urban trees.

According to Fungal Database 53 records of fungus association with this host have been reported worldwide (Farr and Rossman, 2022). Among these, particular attention is given to species belonging to *Botryosphaeriaceae*. In fact, diseases caused by *Botryosphaeriaceae* are drawing the attention of the researchers worldwide, since they are a significant threat to many crops, especially in Mediterranean climates (Moral *et al*., 2019b). *Botryosphaeriaceae* include a large group of diverse fungal species, distributed all over the world. These

fungi are well known as plant pathogens, endophytes, and saprophytes of woody hosts (Slippers and Wingfield, 2007). Due to their role as plant pathogens, these fungal species have been studied for a long time, and their impact on forestry and agricultural production is well known (Phillips *et al*., 2013). *Botryosphaeriaceae* induce severe symptoms, such as branch, shoot, and trunk cankers, and blight fruits and leaves.

Botryosphaeriaceae disease studies on *Ficus* spp., including the cultivated common fig (*F. carica*), have been published worldwide, showing that *Botryosphaeriaceae* and *Diaporthaceae* spp. are involved in complex diseases (Aiello *et al*., 2020; Al-BBedak *et al*., 2018; Banihashemi and Javadi, 2009; Çeliker and Michailides, 2012; El-Atta and Aref, 2013; Elshafie and Ba-Omar, 2002; Giha, 1975; Güney *et al*., 2022; Gusella *et al*., 2020a; Hampson, 1981; Javadi and Banihashemi, 2005; Lima *et al*., 2005; Mayorquin *et al*., 2012; Mirzaee *et al*., 2002; Mohali *et al*., 2017; Ray *et al*., 2010; Rehab *et al*., 2014). *Botryosphaeriaceae* cause polyetic epidemics (2–3 cycles per season); thus, the progress of epidemics may extend for several years (Moral *et al*., 2019b). In addition, *Botryosphaeriaceae*, characterized by a wide host range, can easily jump from one host to another; this is particularly evident in Mediterranean landscapes, where different crops are cultivated nearby (Moral *et al*., 2019b). Especially in the case of urban environments, it must be remembered that dangerous situations are related to the health status of the trees. Therefore, it is important to monitor the health of trees before they become hazardous (Penerbit, 2013). Surveys conducted in the metropolitan area of Catania and Siracusa (Sicily), during 2019 and 2020 revealed many *F. microcarpa* distributed among numerous metropolitan areas, including gardens, public parks, treelined streets, and squares, showing severe symptoms of branch cankers and dieback. The aims of this study were to (i) investigate the etiology of the disease by (ii) characterizing the fungal isolates recovered from

diseased trees based on a multi-loci phylogenetic analysis and (iii) assess their pathogenicity.

3.2 Materials and methods

3.2.1 Surveys and fungal isolation

During the years between 2019 and 2020, surveys were carried out in numerous urban areas of the cities of Catania (Catania province), and Siracusa (Siracusa province), Sicily, where *F. microcarpa* were the most prevalent ornamental trees, including tree-lined streets, gardens, public parks, and squares. Several symptomatic samples obtained from ten plants were collected and brought to the laboratory of the Dipartimento di Agricoltura, Alimentazione e Ambiente, University of Catania, for further investigations. For culture isolation, small sections (0.2 to 0.3 cm^2) of symptomatic tissues (branches and shoots) were surface-disinfected for 1 min in 1.5% sodium hypochlorite, rinsed in sterile water, dried on sterile absorbent paper under laminar hood and placed on potato dextrose agar (PDA, Lickson, Vicari, Italy) amended with 100 mg/liter of streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) (PDAS) to prevent bacterial growth, and then incubated at 25 ± 1°C for 3–5 days until fungal colonies were large enough to be examined. Subsequently, colonies of interest were transferred to fresh PDAS to make pure cultures, and then single-hyphal tip cultures were obtained and maintained on PDAS at 25 ± 1°C. Isolates characterized in this study were stored in the fungal collection of the laboratory with the labels FA, FB, and FM.

3.2.2 Morphological and Molecular Characterization

For the morphological characterization of the pathogens, the length and width of 50 conidia from the 21-day-old colonies of the isolates FM1, FA10, and FA1 grown on PDA were measured using a fluorescence microscope (Olympus-BX61) coupled to an Olympus DP70 digital camera; measurements were captured using software analysis 3.2 (Soft Imaging System GmbH, Münster, Germany). Dimensions are reported as the minimum and maximum in parentheses and the average. Total fungal DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), scraping the mycelium with a sterile scalpel from 5-day-old fungal cultures grown on PDA or malt extract agar (MEA, Oxoid LTD. Basingstoke, Hampshire, England) media. The genomic DNA extracted was visualized on 1% agarose gels (90 V for 40 min) stained with GelRed® (Biotium, Fremont, CA, USA). The quality of the DNA was determined through Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The internal transcriber spacer region (ITS) of the nuclear ribosomal RNA operon was amplified with primers ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White *et al*., 1990); the primers EF1-728F (5′-CAT CGA GAA GTT CGA GAA GG-3′) and EF1-986R (5′-TAC TTG AAG GAA CCC TTA CC-3′) (Carbone and Kohn, 1999) were used to amplify part of the translation elongation factor 1alpha gene (tef1-α); and primer sets Bt2a (5′-GGT AAC CAA ATC GGT GCT TTC-3′) and Bt2b (5′-ACC CTC AGT GTA GTG ACC CTT GGC-3′) (Glass and Donaldson, 1995) were used for the partial beta tubulin (*tub2*). Amplification by polymerase chain reaction (PCR) was performed in a total volume of 25 µL using One Taq® 2X Master Mix with Standard Buffer (BioLabs, New England, NEB), according to the manufacturer's instructions, on an Eppendorf Mastercycler (AG 22331 Hamburg, Germany). The thermal cycle consisted of initial 30 s at 94 ◦C, followed by 35 cycles at 94 ◦C for 30 s, 49 ◦C (ITS), 57–

59 ◦C (*tef1-α*), or 52 ◦C (*tub2*) for 1 min, 68 ◦C for 1 min, and 5 min at 68 ◦C. Regarding *Botryosphaeriaceae*, in total, 45 isolates were sequenced (*tub2*) and only 13 representative isolates were considered for further gene sequencing and phylogenetic analyses. Concerning the *Eutypella*-like species, a total of 7 isolates were sequenced (ITS and *tub2*). PCR products were visualized on 1% agarose gels (90 V for 40 min), purified, and sequenced by Macrogen Inc. (Seoul, South Korea). Forward and reverse DNA sequences were assembled and edited using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar *et al*., 2018) and submitted to GenBank.

3.2.3 Phylogenetic Analysis

Chromatograms were viewed using FinchTV Version 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com (accessed on 16 February 2022)). Sequences were read and edited using MEGAX. Before constructing the phylogenetic tree, BLAST searches were performed using the NCBI nucleotide database (Altschul *et al*., 1990). ITS, *tef1-α*, and *tub2* DNA sequence datasets were aligned using MEGAX, and manual alignments were performed when necessary. A partition-homogeneity test with heuristic search and 1000 homogeneity replicates was performed using PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0a (Sinauer Associates, Sunderland, MA, USA) (Swofford, 2022) to test for discrepancies in the three gene dataset. For comparison, 79 additional sequences were selected according to the recent literature on the *Botryosphaeriaceae* (Bezerra *et al*., 2021; Zhang *et al*., 2021) to be included in the alignment (Table 1). Maximum parsimony analysis (MP) was performed in PAUP v.4.0a. The analysis of the combined dataset (ITS + *tef1-α* + *tub2*) was performed with the heuristic search

function and tree bisection and reconstruction (TBR) as branch-swapping algorithms with the branch-swapping option set to 'best trees' only. Gaps were treated as 'missing', the characters were unordered and of equal weight, and Maxtrees were limited to 100. Tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated. To identify the best-fit model of nucleotide evolution for each gene according to the Akaike information criterion (AIC), MrModeltest v. 2.4 (Nylander, 2004) was used. The maximum likelihood analysis (ML) of the combined genes was performed in GARLI v.0.951 (Zwickl, 2006). For both analyses, clade support was assessed by 1000 bootstrap replicates. *Guignardia philoprina* (CBS 447.68) and *Phyllosticta citricarpa* (CBS 102374) served as the outgroup in both analyses.

3.2.4 Pathogenicity test

Pathogenicity tests were conducted on potted, healthy, 4-year-old *F. microcarpa* plants maintained at room temperature. For each fungal species, one representative isolate was inoculated. Specifically, three plants were used for each isolate, and five inoculation points were chosen along the trunk on each plant (~30 cm distant one from each other). The inoculation site was first surface-disinfected by spraying with 70% ethanol solution, and wounds were made with a sterilized 6-millimeter cork borer after removing the bark, and a mycelium plug (6 mm in diameter) was placed upside down into the plant tissue wound. Wounds were sealed with Parafilm® (Pechney Plastic Packaging Inc., Chicago, IL, USA). In total, 12 additional wounds were inoculated with sterile PDA plugs as controls. Plants were regularly watered. The presence and length of the resulting lesions were recorded two weeks after the inoculation. Lesion length measurements were analyzed in Statistix 10 (Analytical software, FL,

USA, 2013) via analysis of variance (ANOVA), and mean differences were compared with the Fisher's protected least significant difference (LSD) test at $\alpha = 0.05$. In order to fulfil Koch's postulates, re-isolations were carried out on PDAS following the procedure described above. Each re-isolated fungus was identified through the observation of colony characteristics.

3.3 Results

3.3.1 Surveys and Fungal Isolations

Ficus microcarpa growing in a wide range of site conditions (tree-lined streets, gardens, public parks, and squares) have suffered a widespread dieback in Catania and Siracusa provinces. In the public areas where the research was conducted, more than 40 mature *F. microcarpa* trees (20 to 50 years old) showed cankered twigs and branches on approximately 450 plants. The trees still appeared green in part of the canopy, although this was accompanied by parts of branches and shoots that were defoliated and dead (Figures 5A–D and 6A). Sometimes, it was possible to observe new twigs growing below the damaged branches (Figure 5C). The sample consisted of large portions of branches showing severe internal wood discolouration, including of sapwood and the heartwood (Figure 6B–F). Often, the bark appeared cracked and split along the branches (Figure 6A), and internal cankers were sharply demarcated from adjacent, healthy wood (Figure 6B–F). Isolations frequently (>70%) yielded *Botryosphaeriaceae*-like fungi, characterized, as reported by Slippers and Wingfield (2007), by a 'fluffy' mycelium, either white-to-creamy, pigmented 'greenish brown', or gray-to-gray-black. Moreover, with lower frequencies, colonies of *Eutypella*-like species were also isolated from symptomatic tissues.

Figure 5 Symptoms of *Botryosphaeriaceae* disease observed in urban areas on *F. microcarpa*. A, Diseased (left) and healthy (right) plants. B–D, Defoliation and shoot dieback all over the canopy. C, New twigs growing below the dead shoots.

3.3.2 Morphological Characterization and Phylogenetic Analysis

The PCR amplification of the ITS region, *tef1-α*, and *tub2* generated 577 to 581, 273 to 288, and 422 to 446 bp fragments, respectively. The phylogenetic analyses were performed using a dataset of the three concatenated loci. The sequences generated in this study were deposited in GenBank (Table 3). A preliminary comparison of our sequences in GenBank showed our isolates

Figure 6 Internal symptoms. A, Dead branch showing cracking of the outer layers of the bark (upper), healthy branch (lower). B–F, Internal cankers and bark cracked along the branch with diseased tissue sharply demarcated from adjacent, healthy wood. Scale bar B = 15 cm; C = 50 cm; D-F = 20 cm.

belonging to the genera *Botryosphaeria* and *Neofusicoccum*. The *Eutypella*- like species showed high similarity with different *Eutypella* species submitted to GenBank. Since these isolates were excluded from the phylogenetic analyses due to their results in the pathogenicity test, they were identified as *Eutypella* spp. The phylogenetic analyses were then conducted only for the *Botryosphaeriaceae*. The results of the partition-homogeneity test indicated no ($p = 1.00$) significant differences in the three-gene dataset. The MP analysis of the combined dataset showed that of 2921 total characters, 391 were parsimony-informative, 220 were parsimony-uninformative, and 2310 were constant. In total, 100 trees were retained. Tree length was equal to

1098, CI = 0.707, RI = 0.912, RC = 0.644. The best-fit model of nucleotide evolution based on the AIC was GTR + I + G for ITS, GTR + G for *tef1-α*, and HKY + G for *tub2*. The ML analysis showed that of 2921 total characters, 2310 were constant, 475 were parsimony informative, and 136 were autapomorphic. The results of both analyses showed that the isolates FM1-3, FM6 and 7, and FM9 were grouped in the clade of *B. dothidea* (82/88, MP and ML bootstrap support %, respectively), the isolate FA10 grouped within *N. mediterraneum* clade (97/97), and FA1-3, FM8, FB4, and FB6 were grouped with the clade of *N. parvum* (97/98) (Figure 7). The conidia measurements were (18.66)–22.7–(28.34) × (3.61)–4.9–(6.38) for *B. dothidea*, (14.0)–20.0–(27.2) × (4.3)–5.8–(6.8) for *N. mediterraneum*, and (12.78)–15.1–(16.9) × (4.16)–5.3–(7.21) for *N. parvum*.

Table 3 Information on fungal isolates used in the phylogenetic analyses and their corresponding GenBank accession numbers. Isolates in bold are from this study. T= type material.

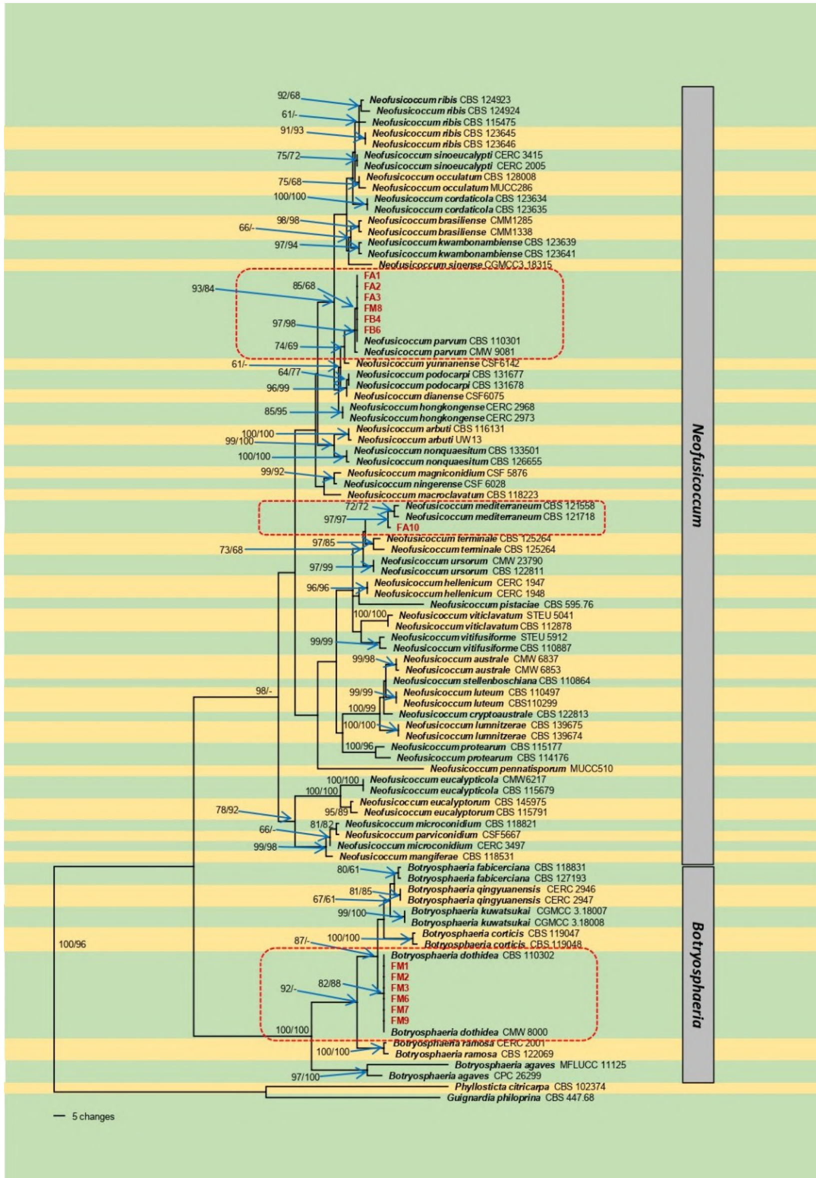
Species	**Isolate ID**	**GenBank accession no.**		
		ITS	*tef1*-α	*tub2*
Botryosphaeria agaves	CBS 133992 = MFLUCC	JX646791	JX646856	JX646841
B. agaves	CBS 141505 = CPC 26299	KX306750	MT592030	MT592463
B. corticis	CBS 119047T	DQ299245	EU017539	EU673107
B. corticis	CBS 119048 = CAP 198	DQ299246	EU017540	MT592464
B. dothidea	CBS 115476 = CMW 8000T	AY236949	AY236898	AY236927
B. dothidea	CBS 110302 = CAP 007	AY259092	AY573218	EU673106
B. dothidea	**FM1**	**OM241975**	**OM262426**	**OM262439**
B. dothidea	**FM2**	**OM241976**	**OM262427**	**OM262440**
B. dothidea	**FM3**	**OM241977**	**OM262428**	**OM262441**

B. dothidea	**FM6**	**OM241978**	**OM262429**	**OM262442**
B. dothidea	**FM7**	**OM241979**	**OM262430**	**OM262443**
B. dothidea	**FM9**	**OM241980**	**OM262431**	**OM262444**
B. fabicerciana	CBS 118831 = CMW 14009	DQ316084	MT592032	MT592468
B. fabicerciana	CBS 127193 = CMW 27094T	HQ332197	HQ332213	KF779068
B. kuwatsukai	CGMCC 3.18007	KX197074	KX197094	KX197101
B. kuwatsukai	CGMCC 3.18008	KX197075	KX197095	KX197102
B. qingyuanensis	CERC 2946 = CGMCC	KX278000	KX278105	KX278209
B. qingyuanensis	CERC 2947 = CGMCC 3.18743	KX278001	KX278106	KX278210
B. ramosa	CERC 2001 = CGMCC	KX277989	KX278094	KX278198
B. ramosa	CBS 122069 = CMW 26167T	EU144055	EU144070	KF766132
Guignardia philoprina	CBS 447.68	FJ824768	FJ824773	FJ824779
Neofusicoccum arbuti	CBS 116131 = AR 4014T	AY819720	KF531792	KF531793
N. arbuti	CBS 117090 = UW13	AY819724	KF531791	KF531794
N. australe	CBS 139662 = CMW 6837T	AY339262	AY339270	AY339254
N. australe	CMW 6853	AY339263	AY339271	AY339255
N. brasiliense	CMM 1285	JX513628	JX513608	KC794030
N. brasiliense	CMM 1338T	JX513630	JX513610	KC794031
N. cordaticola	CBS 123634 = CMW 13992T	EU821898	EU821868	EU821838
N. cordaticola	CBS 123635	EU821903	EU821873	EU821843
N. cryptoaustrale	CBS 122813 = CMW 23785T	FJ752742	FJ752713	FJ752756
N. dianense	CSF6075 = CGMCC3.20082T	MT028605	MT028771	MT028937
N. eucalypticola	CBS 115679 = CMW 6539T	AY615141	AY615133	AY615125
N. eucalypticola	CBS 115766 = CMW 6217	AY615143	AY615135	AY615127
N. eucalyptorum	CBS 115791 = CMW 10125 = BOT 24T	AF283686	AY236891	AY236920

N. eucalyptorum	CBS 145975 = CPC 29337	MT587477	MT592190	MT592682
N. hellenicum	CERC 1947 = CFCC 50067T	KP217053	KP217061	KP217069
N. hongkongense	CERC2973 = CGMCC3.18749T	KX278052	KX278157	KX278261
N. hongkongense	CERC 2968 = CGMCC 3.18748	KX278051	KX278156	KX278260
N. kwambonambiense	CBS 123639 = CMW 14023T	EU821900	EU821870	EU821840
N. kwambonambiense	CBS 123641 = CMW 14140	EU821919	EU821889	EU821859
N. lumnitzerae	CBS 139674 = CMW 41469T	KP860881	KP860724	KP860801
N. lumnitzerae	CBS 139675 = CMW 41228	MT587480	MT592193	MT592685
N. luteum	CBS 110497 = CPC 4594 = CAP 037	EU673311	EU673277	EU673092
N. luteum	CBS 110299 = LM 926 = CAP 002T	AY259091	KX464688	DQ458848
N. macroclavatum	CBS 118223 = CMW 15955 = WAC 12444T	DQ093196	DQ093217	DQ093206
N. magniconidium	CSF5876 = CGMCC3.20077T	MT028612	MT028778	MT028944
N. mangiferae	CBS 118531 = CMW 7024T	AY615185	DQ093221	AY615173
N. mediterraneum	CBS 121558	GU799463	GU799462	GU799461
N. mediterraneum	CBS 121718 = CPC 13137T	GU251176	GU251308	GU251836
N. mediterraneum	**FA10**	**OM241968**	**OM241976**	OM262432
N. microconidium	CERC3497 = CGMCC3.18750T	KX278053	KX278158	KX278262
N. microconidium	CBS 118821 = CMW 13998	MT587497	MT592212	MT592704
N. ningerense	CSF6028 = CGMCC3.20078T	MT028613	MT028779	MT028945
N. nonquaesitum	CBS 126655 = L3IE1 = PD484T	GU251163	GU251295	GU251823
N. nonquaesitum	CBS 133501 = UCR532	MT587498	MT592213	MT592705

N. occulatum	CBS 128008 = MUCC 227T	EU301030	EU339509	EU339472
N. occulatum	MUCC 286 = WAC 12395	EU736947	EU339511	EU339474
N. parvum	CBS 138823 = ICMP 8003 = CMW 9081T	AY236943	AY236888	AY236917
N. parvum	CBS 110301 = CAP 074	AY259098	AY573221	EU673095
N. parvum	**FA1**	**OM241969**	**OM262420**	**OM262433**
N. parvum	**FA2**	**OM241970**	**OM262421**	**OM262434**
N. parvum	**FA3**	**OM241971**	**OM262422**	**OM262435**
N. parvum	**FM8**	**OM241972**	**OM262423**	**OM262436**
N. parvum	**FB4**	**OM241973**	**OM262424**	**OM262437**
N. parvum	**FB6**	**OM241974**	**OM262425**	**OM262425**
N. parviconidium	CSF5667 = CGMCC3.20074T	MT028615	MT028781	MT028947
N. pennatisporum	WAC 13153 = MUCC 510T	EF591925	EF591976	EF591959
N. pistaciae	CBS 595.76T	KX464163	KX464676	KX464953
N. podocarpi	CBS 131677 = CMW 35494	MT587508	MT592223	MT592715
N. podocarpi	CBS 131678 = CMW 35499	MT587509	MT592224	MT592716
N. protearum	CBS 114176 = CPC 1775 = JT 189T	AF452539	KX464720	KX465006
N. protearum	CBS 115177 = CPC 4357	FJ150703	MT592239	MT592731
N. ribis	CBS 115475 = CMW 7772T	AY236935	AY236877	AY236906
N. ribis	CBS 124923 = CMW 28320	FJ900608	FJ900654	FJ900635
N. ribis	CBS 124924T	FJ900607	FJ900653	FJ900634
N. ribis	CBS 123645 = CMW 14058T	EU821904	EU821874	EU821844
N. ribis	CBS 123646 = CMW 14060	EU821905	EU821875	EU821845
N. sinense	CGMCC3.18315T	KY350148	KY817755	KY350154
N. sinoeucalypti	CERC2005 = CGMCC3.18752T	KX278061	KX278166	KX278270

N. sinoeucalypti	CERC3415	KX278063	KX278168	KX278272
N. stellenboschiana	CBS 110864 = CPC 4598	AY343407	AY343348	KX465047
N. terminaliae	CBS 125263 = CMW 26679T	GQ471802	GQ471780	KX465052
N. terminaliae	CBS 125264 = CMW 26683	GQ471804	GQ471782	KX465053
N. ursorum	CBS 122811 = CMW 24480T	FJ752746	FJ752709	KX465056
N. ursorum	CBS 122812 = CMW 23790	FJ752745	FJ752708	KX465057
N. yunnanense	CSF6142 = CGMCC3.20083T	MT028667	MT028833	MT028999
N. viticlavatum	CBS 112878 = CPC 5044 = JM 86T	AY343381	AY343342	KX465058
N. viticlavatum	CBS 112977 = STE-U 5041	AY343380	AY343341	KX465059
N. vitifusiforme	CBS 110887 = CPC 5252 = JM5T	AY343383	AY343343	KX465061
N. vitifusiforme	CBS 121112 = STE-U 5912	EF445349	EF445391	KX465016
Phyllosticta citricarpa	CBS 102374	FJ824767	FJ538371	FJ824778


Neofusicoccum ribis CBS 124923
Neofusicoccum ribis CBS 124924
Neofusicoccum ribis CBS 115475
Neofusicoccum ribis CBS 123645
Neofusicoccum ribis CBS 123646
Neofusicoccum sinoeucalypti CERC 3415
Neofusicoccum sinoeucalypti CERC 2005
Neofusicoccum occulatum CBS 128008
Neofusicoccum occulatum MUCC286
Neofusicoccum cordaticola CBS 123634
Neofusicoccum cordaticola CBS 123635
Neofusicoccum brasiliense CMM1285
Neofusicoccum brasiliense CMM1338
Neofusicoccum kwambonambiense CBS 123639
Neofusicoccum kwambonambiense CBS 123641
Neofusicoccum sinense CGMCC3.18315
FA1
FA2
FA3
FM8
FB4
FB6
Neofusicoccum parvum CBS 110301
Neofusicoccum parvum CMW 9081
Neofusicoccum yunnanense CSF6142
Neofusicoccum podocarpi CBS 131677
Neofusicoccum podocarpi CBS 131678
Neofusicoccum dianense CSF6075
Neofusicoccum hongkongense CERC 2968
Neofusicoccum hongkongense CERC 2973
Neofusicoccum arbuti CBS 116131
Neofusicoccum arbuti UW13
Neofusicoccum nonquaesitum CBS 133501
Neofusicoccum nonquaesitum CBS 126655
Neofusicoccum magniconidium CSF 5876
Neofusicoccum ningerense CSF 6028
Neofusicoccum macroclavatum CBS 118223
Neofusicoccum mediterraneum CBS 121558
Neofusicoccum mediterraneum CBS 121718
FA10
Neofusicoccum terminale CBS 125264
Neofusicoccum terminale CBS 125264
Neofusicoccum ursorum CMW 23790
Neofusicoccum ursorum CBS 122811
Neofusicoccum hellenicum CERC 1947
Neofusicoccum hellenicum CERC 1948
Neofusicoccum pistaciae CBS 595.76
Neofusicoccum viticlavatum STEU 5041
Neofusicoccum viticlavatum CBS 112878
Neofusicoccum vitifusiforme STEU 5912
Neofusicoccum vitifusiforme CBS 110887
Neofusicoccum australe CMW 6837
Neofusicoccum australe CMW 6853
Neofusicoccum stellenboschiana CBS 110864
Neofusicoccum luteum CBS 110497
Neofusicoccum luteum CBS110299
Neofusicoccum cryptoaustrale CBS 122813
Neofusicoccum lumnitzerae CBS 139675
Neofusicoccum lumnitzerae CBS 139674
Neofusicoccum protearum CBS 115177
Neofusicoccum protearum CBS 114176
Neofusicoccum pennatisporum MUCC510
Neofusicoccum eucalypticola CMW6217
Neofusicoccum eucalypticola CBS 115679
Neofusicoccum eucalyptorum CBS 145975
Neofusicoccum eucalyptorum CBS 115791
Neofusicoccum microconidium CBS 118821
Neofusicoccum parviconidium CSF5667
Neofusicoccum microconidium CERC 3497
Neofusicoccum mangiferae CBS 118531
Botryosphaeria fabicerciana CBS 118831
Botryosphaeria fabicerciana CBS 127193
Botryosphaeria qingyuanensis CERC 2946
Botryosphaeria qingyuanensis CERC 2947
Botryosphaeria kuwatsukai CGMCC 3.18007
Botryosphaeria kuwatsukai CGMCC 3.18008
Botryosphaeria corticis CBS 119047
Botryosphaeria corticis CBS 119048
Botryosphaeria dothidea CBS 110302
FM1
FM2
FM3
FM6
FM7
FM9
Botryosphaeria dothidea CMW 8000
Botryosphaeria ramosa CERC 2001
Botryosphaeria ramosa CBS 122069
Botryosphaeria agaves MFLUCC 11125
Botryosphaeria agaves CPC 26299
Phyllosticta citricarpa CBS 102374
Guignardia philoprina CBS 447.68
92/68
61/-
91/93
75/72
75/68
100/100
98/98
66/-
97/94
93/84
85/68
97/98
74/69
61/-
64/77
96/99
85/95
100/100
99/100
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99/92
72/72
97/97
97/85
73/68
97/99
96/96
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98/-
99/99
100/99
100/100
100/96
100/100
100/100
78/92
95/89
81/82
66/-
99/98
80/61
81/85
67/61
99/100
100/100
87/-
100/96
92/-
82/88
100/100
100/100
97/100
5 changes
Neofusicoccum
Botryosphaeria

Figure 7 One of 100 equally parsimonious trees generated from maximum-parsimony analysis of the three-gene (ITS + *tef1-α* + *tub2*) combined dataset from *Botryosphaeriaceae* species. Numbers in front and after the slash represent parsimony and likelihood bootstrap values from 1000 replicates, respectively. Isolated in red were generated in this study. Bar indicates the number of nucleotide changes.

3.3.3 Pathogenicity test

The results of the pathogenicity test showed that all three species of *Botryosphaeriaceae* identified in this study were pathogenic to *F. microcarpa*. Otherwise, the *Eutypella* sp. isolate inoculated did not induce any lesions on the woody tissues, which was similar to the control. For this reason, this species was excluded from the phylogenetic analyses. External discoloration out of the inoculation point was observed after 7 days and all the inoculated trees showed severe wood discoloration after the outer layer of bark was removed (Figure 8A–D). Moreover, young twigs close to the inoculation point rapidly wilted a few days after inoculation. Specifically, among the fungal species, the *N. mediterraneum* isolate FA10 induced the longest lesions (mean 8.10 cm), followed by *N. parvum* isolate FB4 (2.66 cm) and *B. dothidea* isolate FM2 (1.88 cm). All the inoculated species statistically differed from the control ($p < 0.05$) (Figure 9). The colonies that emerged from the re-isolations showed morphological characteristics (color, shape, and mycelium texture) that fulfilled the Koch's postulates.

Figure 8 Results of pathogenicity test after two weeks. A, *Neofusicoccum mediterraneum*. B, *N. parvum*. C, *Botryosphaeria dothidea*. D, Control. Scale bar = 10 cm.

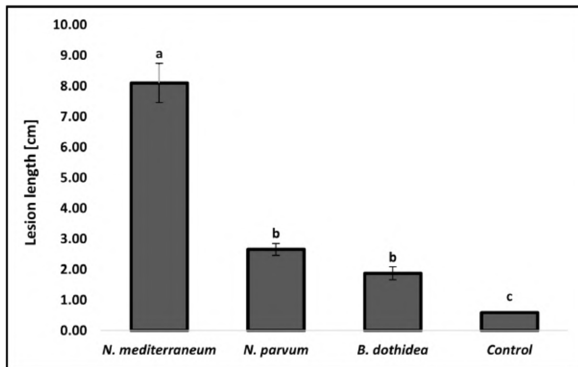

Figure 9 Comparisons of average lesion length (cm) resulting from pathogenicity test among *B. dothidea*, *N. mediterraneum* and *N. parvum* on potted plants. Columns are the means of 15 inoculation points (five per plant) for each fungal species. Control consisted of 12 inoculation points. Vertical bars represent the standard error of the means. Bars topped with different letters indicate treatments that were significantly different according to Fisher's protected LSD test ($\alpha = 0.05$).

3.4 Discussion

The results of our study confirm, for the first time, the presence of three species, *B. dothidea*, *N. mediterraneum*, and *N. parvum*, affecting *F. microcarpa* in Italy. Regarding *Botryosphaeriaceae*, little is known about its association with *F. microcarpa*. According to the U.S. National Fungus Collections Fungal Database (Farr and Rossman, 2022), only a few, old reports describe the association of *Lasiodiplodia theobromae* (as *Botryodiplodia theobromae*) in Pakistan (Ahmad *et al*., 1997) and Egypt (Rehab *et al*., 2014), and *Diplodia fici-retusae* in Taiwan on *Ficus retusa* (synonymous of *F. microcarpa*) (Anonymous, 1979; Sawada, 1959). A commonly reported disease of *F. microcarpa*, as well as other *Ficus* spp., is “sooty canker”, which is caused by *Neoscytalidium dimidiatum* (traditionally reported also as *Hendersonula toruloidea* and *Natrassia mangiferae*). The pathogen, as well as other *Botryosphaeriaceae*, induces cankers and dieback, often accompanied by a powdery mass of black spores (arthroconidia) produced by this species (Al-Bedak *et al*., 2018; Banihashemi and Javadi, 2009; Çeliker and Michailides, 2012; El-Atta and Aref, 2013; Elshafie and Omar, 2002; Gusella *et al*., 2020a; Mirzaee *et al*., 2002; Ray *et al*., 2010; Hodel *et al*., 2009). Recently, in California *B. dothidea*, *N. luteum*, *N. mediterraneum*, and *N. parvum* were reported as causing branch cankers and dieback on *F. microcarpa* trees in Los Angeles County (Mayorquin *et al*., 2012). In recent years, *Botryosphaeriaceae* spp. have been reported attacking many different crops in Italy, and, especially in Sicily, it is well known that these species spread from nurseries to the open field, from ornamental plants to the agricultural ones. Specifically, *B. dothidea* has recently been reported in Sicily on walnut and pistachio (Gusella *et al*. 2020b, 2021b). Moreover, *N. mediterraneum* and *N. parvum* have been reported as highly aggressive pathogens among the

Botryosphaeriaceae in Sicily (Aiello *et al*., 2020; Gusella *et al*., 2020b, 2021a; Ismail *et al*., 2013). In addition, *N. mediterraneum* was the most encountered species in Sicilian pistachio orchards (Gusella *et al*., 2021). From this and previous studies conducted in Sicily, it emerged that *Botryosphaeriaceae* spp., and especially the species described in this study, are easily encountered in different hosts and landscapes. Regarding the ecology of these fungi, it is well known that they are also endophytes on many hosts (Slippers and Wingfield, 2007), often coexisting in the same tissues (Luo *et al.,* 2021) and forming long latent infections (Luo *et al*., 2017, 2019). This must be taken into serious consideration, since many infections can spread from nurseries (as latent infections) to open fields. Recently, studies conducted in California on latent infections on nut crops helped us to properly quantify these pathogens using real-time PCR assays (Luo *et al*., 2017, 2019, 2020). The ability of these fungi to disperse their spores (conidia) by wind, rain, and insects (Moral *et al*., 2019b) in conjunction with intercontinental human movements with no adequate quarantine strategies led them to easily spread all over the world (Slippers *et al*., 2017), as demonstrated for *N. parvum*, the most adapted organism, which is detected from the north to the south, excluding boreal forests and montane grasslands (Batista *et al*., 2021). Many factors can be involved in the ability of some *Botryosphaeriaceae* species to jump from one host to another, meaning that they are more virulent than other species. Among these, a recent study (Garcia *et al*., 2021) revealed how some groups of taxa, such as *Botryosphaeria*, *Lasiodiplodia*, and *Neofusicoccum*, show an expansion of certain clades of gene families involved in the pathogenesis. Specifically, in the *Botryosphaeria* and *Neofusicoccum* genomes, an expansion of secreted cell-wall-degrading enzymes (CAZymes) was observed (Garcia *et al*., 2021). It is no surprise that the species identified in this study also occurred on other taxonomically distant hosts in Sicily. Batista *et al*. (2021) showed that

B. dothidea is associated with 403 hosts in 66 countries, and *N. parvum* with 223 hosts in 50 countries. In recent decades, in Sicily, a relevant increase was observed in *Botryosphaeriaceae* in nurseries, as well as in open fields (Polizzi G., unpublished data). *Botryosphaeriaceae* disease expression is strongly related to stresses due to factors other than the *Botryosphaeriaceae* infection itself (Blodgett and Stanzosz, 1995; Schoeneweiss, 1981; Swart and Wingfield, 1991). Related to this, it should be noticed that climate change contributes to additional stress or pressure on woody plants through extreme weather conditions or the expansion of pathogens' host ranges (Slippers and Wingfield, 2007). In fact, climate change affects the dynamics of fungal populations, in terms of biology and ecology (Swart and Wingfield, 1991). Gange *et al*. (2011) conducted a study in the UK on the species *Auricularia auricula-judae*, demonstrating an alteration in the phenology (the earlier appearance of fruit bodies and a longer fruiting period) and an expansion of the host range consistent with a response to observed warming trends in the climate, also suggesting that climate change affected the interactions between wood-inhabiting fungi. Combative interactions are considered the main drivers of fungal community development in decaying wood (Boddy, 2000; Boddy and Heilmann-Clausen, 2008), and these can be strongly affected by temperature, water potential, gaseous regime, and resource size (Boddy, 2000; Toljander *et al*., 2006; Woodward and Boddy, 2008). All these factors contribute to making *Botryosphaeriaceae* disease severe and ubiquitous, compared with otherwise "mild diseases" (Desprez-Loustau *et al*., 2006). Urban areas, which are even less investigated than agricultural ones, must be considered crucial routes of introduction and dissemination for *Botryosphaeriaceae* (Lopes *et al*., 2016). It is well known that stressed trees are much more predisposed to *Botryosphaeriaceae* disease (Mehl *et al*., 2013; Slippers and Wingfield, 2007), and this should be taken into careful consideration regarding ornamental trees in urban landscapes. In fact,

trees grown in urban areas can also be considered more exposed to stress factors (Tubby and Webber 2010), and thus more susceptible to *Botryosphaeriaceae* disease. This could represent a serious threat in urban areas, not only in terms of aesthetic damage, but mostly in terms of public safety. In relation to these predisposing factors, we ascertained during our investigation that *F. microcarpa* trees grown in the urban areas of Catania and Siracusa provinces were severely and improperly pruned, especially during the humid seasons. In order to avoid the spread of *Botryosphaeriaceae* species, some recommendations should be taken into serious consideration. Since it is known that both rainfall and fog (Kuntzmann *et al*., 2009; Urbez-Torres *et al*., 2010) positively affect the release of *Botryosphaeriaceae* spores, farmers or pruning crews should not prune when rain is forecasted or with dense fog to avoid the contamination of fresh wounds by *Botryosphaeriaceae* (Moral *et al*., 2019a). Moreover, recommendations as to pruning type depend on the tree species, which is why trained pruning crews should be selected for this crucial practice. As demonstrated on pistachio, Botryosphaeria panicle and shoot blight were reduced by 50–60% by trained pruning crews compared to the disease levels in trees pruned by unspecialized crews (Holtz, 2002). Furthermore, in California, field experiments conducted on *F. carica* affected by fig limb dieback demonstrated that pruning 5 cm below the canker successfully removed the pathogen from the tissues (Gusella *et al*., 2021b). Regarding trained pruning crews, it is crucial that workers disinfect their pruning tools, since these could easily transmit inoculum (spores, mycelium, and fruit bodies) from one tree to another. As demonstrated on walnut, pathogen spores were transferred from the chainsaws to the agar media, whereas *Botryosphaeriaceae* species were not found when the chainsaws were disinfected with a 2% dilution of vinegar or commercial household bleach (T.J. Michailides, unpublished data/personal communication). In addition, the usage of biological

control agents as protectants for pruning wounds, especially in urban areas, should be considered. Encouraging results have been obtained on other crops, such as almond and grapevine treated with Trichoderma-based formulants against canker pathogens (Berbegal *et al*., 2020; Holland *et al*., 2021; Pertot *et al*., 2016). Further investigations need to be conducted in this direction. Good agronomic practices and, possibly, the usage of biocontrol agents, can help us to control *Botryosphaeriaceae* disease in urban areas. To our knowledge, this is the first study of *Botryosphaeriaceae* disease on *F. microcarpa* in Europe.

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4. Experimental part 3: *Lasiodiplodia citricola*, a new causal agent of *Acacia* spp. dieback

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4.1 New Disease Report

Acacia species are woody perennial trees belonging to the family *Mimosaceae* and native to Australia. In summer 2021, unusual necrotic sunken lesions and wood discolouration were observed at the stem level both in the rootstock and in the scion, as well as the graft union on young *A. dealbata* plants grafted on *A. retinodes* in a nursery in Milazzo (north-western Sicily, Italy). In addition, gumming from cracking of the bark and shoot blight were observed (Figure 10). The disease incidence was estimated around 20% on approximately 2,500 plants. Symptoms were different from those previously observed on *A. retinodes* in Sicily (caused by *Cylindrocladium pauciramosum*) (Polizzi and Catara, 2001).
Diseased vascular stem tissue segments were cut and transferred onto potato dextrose agar medium (PDA) and incubated at 25◦C in the dark for seven days. Colonies resembling *Botryosphaeriaceae* was consistently isolated (Figure 11). Pycnidia grown on pine needles agar (PNA) were observed using a stereoscopic microscope and harvested. Conidia were hyaline, non-septate, and ellipsoid. The length × width of 50 conidia were 22.52 × 12.54 μm (Figure 12). The internal transcriber spacer region (ITS) was amplified with primers ITS5/ITS4 (White *et al.*, 1990), and primers Bt2a/Bt2b were used for the partial β-tubulin (*tub2*) (Glass and Donaldson, 1995). Resultant sequences were deposited in GenBank with Accession Nos. OM891495-OM891502 for the ITS and OM918761-OM918768 for *tub2*. For comparison, 66 additional sequences were selected and aligned according to recent literature on the *Botryosphaeriaceae* (Zhang *et al.*, 2021). Maximum parsimony analysis of *tub2* region was performed in PAUP v.4.0a (Swofford, 2002). Clade support was assessed by 1,000 bootstrap replicates. *Neodeightonia phoenicum* served as an outgroup. Our isolates clustered within the group of *Lasiodiplodia vaccinii* (65%

bootstrap value) (ex-type CGMCC 3.19022), described by Zhao *et al*. (2019), and now synonymised with *L. citricola* (Zhang *et al*., 2021). Based on both morphological and molecular results, the organism was identified as *L. citricola* (Figure 13). Pathogenicity tests were conducted on three one-year-old *A. retinodes* rootstocks and three one-year-old *A. dealbata* grafted pot plants. The inoculum consisted of a mycelial plug from a seven-day- old culture inserted at three different inoculation points on each plant wounded with a cork borer (15 cm from each other) along the stem. Controls were inoculated with sterile PDA. After one week, symptoms of wood discolouration and dieback were observed and then 70% of inoculated plants died (Figure 14). Once symptoms appeared, re-isolations were conducted as described above showing colonies resembling *Lasiodiplodia*, therefore Koch's postulates were fulfilled.

To our knowledge, this is the first report of *L. citricola* causing shoot blight and canker on *A. retinodes* and *A. dealbata* worldwide. High humidity is an important factor for spore dispersion, therefore sprinkler irrigation should be avoided in greenhouses. Moreover, grafting is a crucial step, through which infections can easily occur, especially in the case of *L. citricola*, which has previously been associated with graft failure (Chen *et al*., 2013). Disease management starts with sanitation of the tools and of the propagation material to avoid infection with *Botryosphaeriaceae*.

Figure 10 Symptoms of A, stem canker; B, canker at the graft union; C, gummosis and D, internal necrosis of the stem, observed on trees in a in a nursery in Milazzo, Italy.

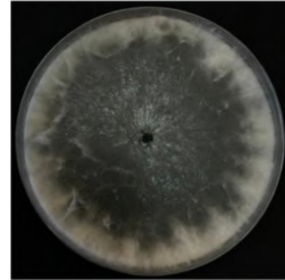

Figure 11 Colony of *Lasiodiplodia citricola* grown on potato dextrose agar medium after 7 days.

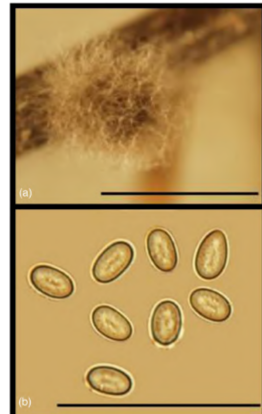

Figure 12 A, Conidioma on pine needle (Bar=2mm) and B, conidia (Bar = 50 μm).

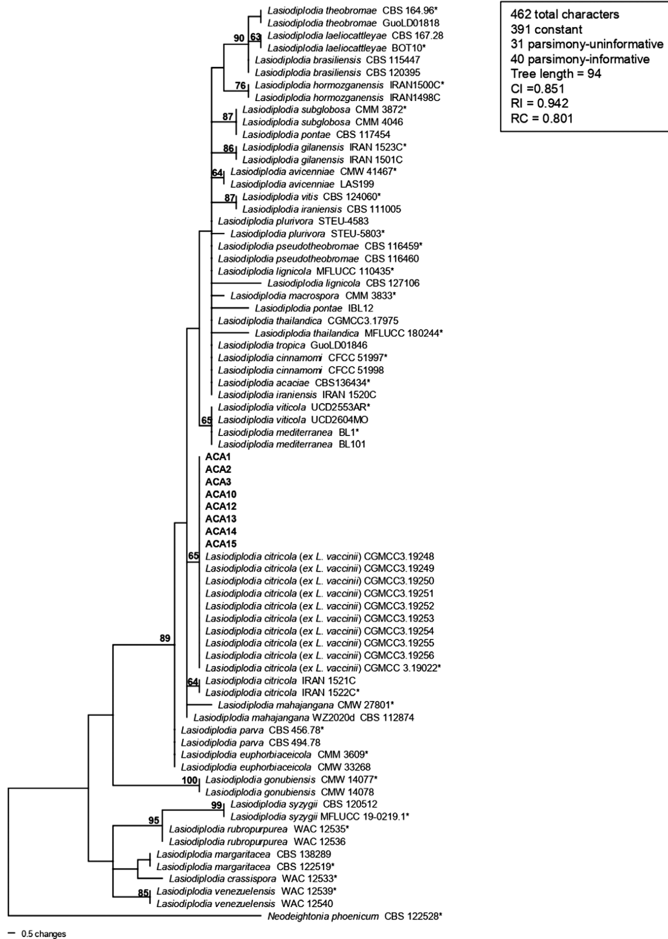

Figure 13 Phylogenetic tree derived from the maximum parsimony analysis of *tub2* gene region. Numbers close to the nodes represent parsimony bootstrap values from 1,000 replicates. The asterisk indicates type material. Isolates in bold are generated in this study. Bar indicates the number of nucleotide changes.

Figure 14 Pathogenicity test. A, twig dieback. B, pycnidia at the inoculation point. C, stem of inoculated plant (left), control (right).

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5. Other research activities: *Neopestalotiopsis siciliana* sp. nov. and *N. rosae* Causing Stem Lesion and Dieback on Avocado Plants in Italy

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5.1 Introduction

Avocado (*Persea americana* Mill.) is a tree native to Central America and is widely cultivated, especially in tropical and subtropical regions. In the recent years, the consumption and new plantings of this tropical fruit are increasing world-wide. Global top producers (1000 metric tons unit, year 2020) include Mexico (2390), Colombia (876), and the Dominican Republic (676) (FAO Data, 2022). Of European countries, Spain is the main avocado producer (99), followed by Greece (10) (FAO Data, 2022). In southern Italy, specifically in Sicily, avocado represents an emerging crop in terms of economic opportunity for the growers. In recent years, an increasing consumer interest towards tropical fruits has been observed. Within this new trend, avocado fruit presents great potential due to its high nutritional value and peculiar quality characteristics to achieve requirements desired by consumers (Migliore *et al*., 2018). In Italy, since this crop is emerging, few studies have been conducted on the phytopathological situation. Regarding the fungal diseases affecting avocado, several fungal taxa have been reported associated with different symptoms (Zentmyer, 1994), and some of those pose a severe threat for its production around the world. Among these, *Phytophthora cinnamomi* is considered the most important and widely spread pathogen of avocado, causing significant economic losses (Zentmyer, 1980). *Rosellinia necatrix*, the causal agent of white root rot, is a serious threat in the Mediterranean area and is considered the main cause of avocado losses in Spain (López-Herrera *et al*., 1992), and recently it has also been reported in Italy (Fiorenza *et al*., 2021). In Italy, as well as around the world (Parkinson *et al*., 2017), several species belonging to the *Nectriaceae* family (i.e., *Cylindrocladiella peruviana*, *Ilyonectria macrodidyma*, and *Pleiocarpon algeriense*) have been studied in the last few years, and have been associated with

root and crown rot (Aiello *et al*., 2020; Vitale *et al*., 2012). Several fungal species belonging to *Botryosphaeriaceae* and *Diaporthaceae* families are known to be causal agents of branch canker and fruit stem-end rot on avocado (Guarnaccia *et al*., 2016; Hartill and Everett, 2002; McDonald and Eskalen, 2011; Menge and Ploetz, 2003). In addition, in 2018, a new species named *Neocosmospora perseae* was described as causing trunk cankers in Italy and more recently in Greece (Guarnaccia *et al*., 2018, 2021). *Colletotrichum* spp. are reported as important pre- and post- harvest pathogens (Freeman *et al*., 1998; Kimaru *et al*., 2018; Sharma *et al*., 2017; Silva-Rojas and Ávila-Quezada, 2011). Moreover, pestalotioid fungi, known as major agents of leaf spot diseases, were also reported on avocado (Maharachchikumbura *et al*., 2014b; Valencia *et al*., 2011; Vitale *et al*., 2005). During December of 2020, surveys conducted in a commercial avocado orchard in Sicily (Italy) revealed the presence of young plants showing external symptoms of dieback and stem lesions on scion at the grafting point or slightly above. Since avocado is considered an emerging crop in Italy, especially in the southern regions, it is crucial to investigate the etiology of the diseases that could represent an important limiting factor. The aim of the present study was to investigate the etiology of the symptoms observed in the field and to identify the causal fungal agents to species by morphology and molecular data.

5.2 Materials and Methods

5.2.1 Isolation and Morphological Characterization

Samples were collected in the field on approximately 20 plants of *Persea americana* cv. "Hass" grafted on "Zutano", randomly selected, and were brought to the Plant Pathology laboratory of the Department of Agriculture, Food, and Environment at the University

of Catania for further investigations. One hundred small sections (5 mm × 5 mm) of the stems were surface disinfected for 1 min in 1.5% sodium hypochlorite (NaOCl), rinsed in sterile distilled water, dried on sterile absorbent paper, and placed on potato dextrose agar (PDA; Lickson, Vicari, Italy) amended with 100 mg/L of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA) to prevent bacterial growth, and then incubated at 25 ± 1 °C for five to seven days. Single-spore isolates were obtained from conidia produced in pure cultures grown on PDA. To determine the effect of temperature on mycelial growth and the optimal growth temperature, the representative isolates AC46 and AC50 were cultured on PDA for further assays. After seven days of incubation at 25 °C, 5 mm diam. mycelial plugs were transferred from the edge of the colonies to the center of PDA Petri plates. The plates were incubated at 5–10–15–20–25–30–35 °C in the dark. Three Petri plates were used for each temperature as replicates. The experiment was repeated once. After seven days of incubation, two perpendicular diameters of the colonies were measured with a scale ruler. The isolates used in this study are maintained in the culture collection of the Department of Agriculture, Food, and Environment, University of Catania. Moreover, representative isolates were deposited at the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, the Netherlands, and dried sporulating cultures were deposited as vouchers in the fungarium of the Department of Botany and Biodiversity Research, University of Vienna (WU-MYC).
Study of macromorphology of conidiomata was performed by using a Nikon SMZ 1500 stereomicroscope equipped with a Nikon DS-U2 digital camera or with a Keyence VHX- 6000 digital microscope (Mechelen, Belgium). Microscopic preparations were mounted in water. For light microscopy, a Zeiss Axio Imager.A1 compound microscope (Oberkochen, Germany), equipped with Nomarski differential interference contrast (DIC) optics and a Zeiss Axiocam 506 color digital camera, was used. Photographs and measurements

were taken by using the NIS-Elements D v. 3.0 or Zeiss ZEN Blue Edition software. For certain images of conidia, the stacking software Zerene Stacker version 1.04 (Zerene Systems LLC, Richland, WA, USA) was used. Measurements are reported as maxima and minima in parentheses and the mean plus and minus the standard deviation of a number of measurements given in parentheses.

5.2.2 DNA Extraction, PCR, and Phylogenetic Analysis

The genomic DNA was extracted from surface mycelium scraped off from pure cultures, using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). The following three loci were amplified and sequenced: the complete internal transcribed spacer region (ITS1-5.8S-ITS2) with primers ITS5 and ITS4 (White *et al*., 1990); a ca. 0.5 kb fragment of the translation elongation factor 1-alpha (*tef1-α*) gene with primers EF1-728F (Carbone and Kohn, 1999) and *tef1-α* D_iR (Voglmayr *et al*., 2018); and a ca. 0.95 kb fragment of the beta-tubulin (*tub2*) gene with primer pairs T1 (O'Donnell and Cigelnik, 1997) and BtHV2r (Voglmayr *et al*., 2016). The PCR products were purified using an enzymatic PCR cleanup (Werle *et al*., 1994), as described by Voglmayr and Jaklitsch (2008), and sequenced in both directions by Macrogen Inc. (Seoul, South Korea) or at the Department of Botany and Biodiversity Research, University of Vienna, using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems, Warrington, UK) and the original PCR primers; sequencing was performed on an automated DNA sequencer (3730xl Genetic Analyser, Applied Biosystems). The DNA sequences generated were assembled with Lasergene SeqMan Pro (DNASTAR, Madison, WI, USA). Sequences generated during the present study were deposited in Genbank (Table 4). The newly generated sequences

were aligned to a representative matrix of *Neopestalotiopsis*, selecting two species of *Pestalotiopsis* as an outgroup. For *Neopestalotiopsis*, all 70 accepted species were included in the matrix, preferentially with ex-type sequences. The GenBank accession numbers of sequences used in these analyses are given in Table 4.

Table 4. Information of fungal isolates used in the phylogenetic analysis and corresponding GenBank accession numbers. Isolates in bold are from this study.

Species	Strain[a]	Host/Substrate	Origin	GenBank Accession Numbers			Reference
				ITS	*tef1-α*	*tub2*	
Neopestalotiopsis acrostichi	MFLUCC 17-1754[T]	*Acrostichum aureum*	Thailand	MK764272	MK764316	MK764338	Norphanphour *et al*. (2019)
N. alpapicalis	MFLUCC 17-2544[T]	*Rhizophora mucronata*	Thailand	MK357772	MK463547	MK463545	Kumar *et al*. (2019)
N. aotearoa	CBS 367.54[T]	Canvas	New Zealand	KM199369	KM199526	KM199454	Maharachchikumbura *et al*. (2012b)
N. asiatica	MFLUCC 12-0286[T]	Unidentified tree	China	JX398983	JX399049	JX399018	Maharachchikumbura *et al*. (2012)
N. australis	CBS 114159[T]	*Telopea* sp.	Australia	KM199348	KM199537	KM199348	Maharachchikumbura *et al*. (2014b)
N. brachiata	MFLUCC 17-1555[T]	*Rhizophora apiculata*	Thailand	MK764274	MK764318	MK764340	Norphanphour *et al*. (2019)
N. brasiliensis	COAD 2166[T]	*Psidium guajava*	Brazil	MG686469	MG692402	MG692400	Bezerra *et al*. (2018)
N. camelliae-oleiferae	CSUFTCC81[T]	*Camellia oleifera*	China	OK493585	OK507955	OK562360	Li *et al*. (2021)
N. cavernicola	KUMCC 20-0269[T]	Cave rock surface	China	MW545802	MW550735	MW557596	Liu *et al*. (2021)
N. chiangmaiensis	MFLUCC 18-0113[T]	Dead leaves	Thailand	-	MH388404	MH412725	Tibpromma *et al*. (2018)
N. chrysea	MFLUCC 12-0261[T]	*Pandanus* sp.	China	JX398985	JX399051	JX399020	Maharachchikumbura *et al*. (2012)
N. clavispora	MFLUCC 12-0281[T]	*Magnolia* sp.	China	JX398979	JX399045	JX399014	Maharachchikumbura *et al*. (2012)

N. cocoes	MFLUCC 15-0152^T	*Cocos nucifera*	Thailand	NR_156312	KX789689	-	Norphanphour *et al.* (2019)
N. coffeae-arabicae	HGUP 4019^T	*Coffea arabica*	China	KF412649	KF412646	KF412643	Song *et al.* (2013)
N. cubana	CBS 600.96^T	Leaf litter	Cuba	KM199347	KM199521	KM199438	Maharachchikumbura *et al.* (2014b)
N. dendrobii	MFLUCC 14-0106^T	*Dendrobium cariniferum*	Thailand	MK993571	MK975829	MK975835	Ma *et al.* (2019)
N. drenthii	BRIP 72264a^T	*Macadamia integrifolia*	Australia	MZ303787	MZ344172	MZ312680	Prasannath *et al.* (2021)
N. egyptiaca	CBS 140162^T	*Mangifera indica*	Egypt	KP943747	KP943748	KP943746	Crous *et al.* (2015)
N. ellipsospora	MFLUCC 12-0283^T	Dead plant materials	China	JX398980	JX399047	JX399016	Maharachchikumbura *et al.* (2012)
N. eucalypticola	CBS 264.37^T	*Eucalyptus globulus*	-	KM199376	KM199551	KM199431	Maharachchikumbura *et al.* (2014b)
N. eucalyptorum	CBS 147684^T	*Eucalyptus globulus*	Portugal	MW794108	MW805397	MW802841	Diogo *et al.* (2021)
N. foedans	CGMCC 3.9123^T	Mangrove plant	China	JX398987	JX399053	JX399022	Maharachchikumbura *et al.* (2012)
N. formicidarum	CBS 362.72^T	Dead *Formicidae* (ant)	Ghana	KM199358	KM199517	KM199455	Maharachchikumbura *et al.* (2014b)
N. guajavae	FMBCC 11.1^T	*Psidium guajava*	Pakistan	MF783085	MH460868	MH460871	Ul Haq *et al.* (2021)
N. guajavicola	FMBCC 11.4^T	*Psidium guajava*	Pakistan	MH209245	MH460870	MH460873	Ul Haq *et al.* (2021)
N. hadrolaeliae	COAD 2637^T	*Hadrolaelia jongheana*	Brazil	MK454709	MK465122	MK465120	Freitas *et al.* (2019)
N. hispanica	CBS 147686^T	*Eucalyptus globulus*	Portugal	MW794107	MW805399	MW802840	Diogo *et al.* (2021)
N. honoluluana	CBS 114495^T	*Telopea* sp.	USA	KM199364	KM199548	KM199457	Maharachchikumbura *et al.* (2014b)
N. hydeana	MFLUCC 20-0132^T	*Artocarpus heterophyllus*	Thailand	MW266069	MW251129	MW251119	Huanlauek *et al.* (2021)

N. iberica	CBS 147688^T	*Eucalyptus globulus*	Portugal	MW794111	MW805402	MW802844	Diogo *et al*. (2021)
N. iranensis	CBS 137768^T	*Fragaria* × *ananassa*	Iran	KM074048	KM074051	KM074057	Ayoubi and Soleimani (2015)
N. javaensis	CBS 257.31^T	*Cocos nucifera*	Indonesia	KM199357	KM199548	KM199457	Maharachchikumbura *et al*. (2014b)
N. longiappendiculata	CBS 147690^T	*Eucalyptus globulus*	Portugal	MW794112	MW805404	MW802845	Diogo *et al*. (2021)
N. lusitanica	CBS 147692^T	*Eucalyptus globulus*	Portugal	MW794110	MW805406	MW802843	Diogo *et al*. (2021)
N. macadamiae	BRIP 63737c^T	*Macadamia integrifolia*	Australia	KX186604	KX186627	KX186654	Akinsanmi *et al*. (2017)
N. maddoxii	BRIP 72266a^T	*Macadamia integrifolia*	Australia	MZ303782	MZ344167	MZ312675	Prasannath *et al*. (2021)
N. magna	MFLUCC 12-0652^T	*Pteridium* sp.	France	KF582795	KF582791	KF582793	Maharachchikumbura *et al*. (2014a)
N. mesopotamica	CBS 336.86^T	*Pinus brutia*	Turkey	KM199362	KM199555	KM199441	Maharachchikumbura *et al*. (2014b)
N. musae	MFLUCC 15-0776^T	*Musa* sp.	Thailand	NR_156311	KX789685	KX789686	Norphanphour *et al*. (2019)
N. natalensis	CBS 138.41^T	*Acacia mollissima*	South Africa	NR_156288	KM199552	KM199466	Maharachchikumbura *et al*. (2014a)
N. nebuloides	BRIP 66617^T	*Sporobolus jacquemontii*	Australia	MK966338	MK977633	MK977632	Crous *et al*. (2020)
N. olumideae	BRIP 72273a^T	*Macadamia integrifolia*	Australia	MZ303790	MZ344175	MZ312683	Prasannath *et al.* (2021)
N. pandanicola	KUMCC 17-0175^T	*Pandanus* sp.	China	-	MH388389	MH412720	Tibpromma *et al*. (2018)
N. pernambucana	URM 7148-01^T	*Vismia guianensis*	Brazil	KJ792466	KU306739	-	Silvério *et al*. (2016)
N. perukae	FMBCC 11.3^T	*Psidium guajava*	Pakistan	MH209077	MH523647	MH460876	Ul Haq *et a*l. (2021)
N. petila	MFLUCC 17-1738^T	*Rhizophora apiculata*	Thailand	MK764276	MK764320	MK764342	Norphanphour *et a*l. (2019)

N. phangngaensis	MFLUCC 18-0119T	*Pandanus* sp.	Thailand	MH388354	MH388390	MH412721	Tibpromma *et al*. (2018)
N. piceana	CBS 394.48T	*Picea* sp.	UK	KM199368	KM199527	KM199453	Maharachchikumbura et al. (2014b)
N. protearum	CBS 114178T	*Leucospermum cuneiforme*	Zimbabwe	JN712498	KM199542	KM199463	Crous *et al*. (2011)
N. psidii	FMBCC 11.2T	*Psidium guajava*	Pakistan	MF783082	MH460874	MH477870	Ul Haq et al. (2021)
N. rhapidis	GUCC 21501T	*Rhododendron simsii*	China	MW931620	MW980442	MW980441	Yang *et al*. (2021)
N. rhizophorae	MFLUCC 17-1551T	*Rhizophora mucronata*	Thailand	MK764277	MK764321	MK764343	Norphanphour *et a*l. (2019)
N. rhododendri	GUCC 21504T	*Rhododendron simsii*	China	MW979577	MW980444	MW980443	Yang *et a*l. (2021)
N. rosae	CBS 101057T	*Rosa* sp.	New Zealand	KM199359	KM199523	KM199429	Maharachchikumbura *et al*. (2014b)
N. rosae	CBS 124745	*Paeonia suffruticosa*	USA	KM199360	KM199524	KM199430	Maharachchikumbura *et al*. (2014b)
N. rosae	CRM-FRC	*Fragaria × ananassa*	Mexico	MN385718	MN268532	MN268529	Rebollar-Alviter *et al*. (2020)
N. rosae	**AC50**	***Persea americana***	**Italy**	**ON117810**	**ON107276**	**ON209165**	**this study**
N. rosicola	CFCC 51992T	*Rosa chinensis*	China	KY885239	KY885243	KY885245	Norphanphour *et al*. (2019)
N. samarangensis	MFLUCC 12-0233T	*Syzygium samarangense*	Thailand	JQ968609	JQ968611	JQ968610	Norphanphour *et al*. (2019)
N. saprophytica	MFLUCC 12-0282T	*Magnolia* sp.	China	JX398982	JX399048	JX399017	Maharachchikumbura *et a*l. (2014b)
N. scalabiensis	CAA1029T	*Vaccinium corymbosum*	Portugal	MW969748	MW959100	MW934611	Santos *et al*. (2022
N. sichuanensis	CFCC 54338T	*Castanea mollissima*	China	MW166231	MW199750	MW218524	Jiang *et al*. (2021)
N. siciliana	**AC46**	***Persea americana***	**Italy**	**ON117813**	**ON107273**	**ON209162**	**this study**

N. siciliana	**AC48**	***Persea americana***	**Italy**	**ON117812**	**ON107274**	**ON209163**	**this study**
N. siciliana	**AC49**	***Persea americana***	**Italy**	**ON117811**	**ON107275**	**ON209164**	**this study**
N. sonneratiae	MFLUCC 17-1745^T	*Sonneronata alba*	Thailand	MK764280	MK764324	MK764346	Norphanphour *et al*. (2019)
N. sp.	TAP18N001	*Eriobotrya japonica*	Japan	LC427126	LC427128	LC427127	Nozawa *et al*. (2020)
N. sp.	TAP18N006	*Eriobotrya japonica*	Japan	LC427141	LC427143	LC427142	Nozawa *et al*. (2020)
N. sp.	TAP18N016	*Eriobotrya japonica*	Japan	LC427168	LC427170	LC427169	Nozawa *et al*. (2020)
N. sp.	TAP18N021	*Eriobotrya japonica*	Japan	LC427183	LC427185	LC427184	Nozawa *et al*. (2020)
N. steyaertii	IMI 192475^T	*Eucalytpus viminalis*	Australia	KF582796	KF582792	KF582794	Maharachchikumbura *et al*. (2014a)
N. surinamensis	CBS 450.74^T	Soil under *Elaeis guineensis*	Suriname	KM199351	KM199518	KM199465	Maharachchikumbura *et al*. (2014b)
N. thailandica	MFLUCC 17-1730^T	*Rhizophora mucronata*	Thailand	MK764281	MK764325	MK764347	Norphanphour *et al*. (2019)
N. umbrinospora	MFLUCC 12-0285^T	unidentified plant	China	JX398984	JX399050	JX399019	Maharachchikumbura *et a*l. (2012)
N. vaccinii	CAA1059^T	*Vaccinium corymbosum*	Portugal	MW969747	MW959099	MW934610	Santos *et a*l. (2022)
N. vacciniicola	CAA1055^T	*Vaccinium corymbosum*	Portugal	MW969751	MW959103	MW934614	Santos *et al*. (2022)
N. vheenae	BRIP 72293a^T	*Macadamia integrifolia*	Australia	MZ303792	MZ344177	MZ312685	Prasannath *et al.* (2021)
N. vitis	MFLUCC 15-1265^T	*Vitis vinifera*	China	KU140694	KU140676	KU140685	Jayawardena *et al*. (2016)
N. zakeelii	BRIP 72282a^T	*Macadamia integrifolia*	Australia	MZ303789	MZ344174	MZ312682	Prasannath *et al*. (2021)
N. zimbabwana	CBS 111495^T	*Leucospermum cunciforme*	Zimbabwe	MH554855	KM199545	KM199456	Maharachchikumbura *et al*. (2014b)
Pestalotiopsis colombiensis	CBS 118553^T	*Eucalyptus grandis* × *urophylla*	Colombia	KM199307	KM199488	KM199421	Maharachchikumbura *et al*. (2014b)

Pestalotiopsis diversiseta	MFLUCC 12-0287[T]	Dead plant material	China	NR_120187	JX399073	JX399040	Maharachchikumbura *et al.* (2012)

BRIP: Queensland Plant Pathology Herbarium, Australia; CAA: Personal culture collection of Artur Alves, Department of Biology, University of Aveiro; CBS: Culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CFCC: China Forestry Culture Collection Center, Research Institute of Forest Ecology, Environment and Protection, Beijing, China; CGMCC: China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; COAD: Culture collection of Coleção Octávio Almeida Drummond of the Universidade Federal de Viçosa, Viçosa, Brazil; CRM: Universidad Autónoma Chapingo, Centro Regional Morelia, Morelia, Michoacán, México; CSUFTCC: Central South University of Forestry and Technology culture collection, Hunan, China; FMBCC: Fungal Molecular Biology Laboratory Culture Collection, University of Agriculture Faisalabad, Pakistan; GUCC: Department of Plant Pathology culture collection, Agriculture College, Guizhou University, China; HGUP: Plant Pathology Herbarium of Guizhou University, Guizhou, China; IMI: Culture collection of CABI Europe UK Centre, Egham, UK; KUMCC: Culture collection of Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China; MFLUCC: Mae Fah Luang University culture collection, Chiang Rai, Thailand; TAP: Culture collection of Tamagawa University, Tokyo, Japan; URM: The Father Camille Torrend Herbarium, Pernambuco, Brazil. Ex-type strains are labeled with T. 2 ITS: internal transcribed spacer; TEF1: translation elongation factor 1-α; TUB2: β-tubulin. N/A: Not available

Sequence alignments for phylogenetic analyses were produced with the server version of MAFFT (http://mafft.cbrc.jp/alignment/server/, accessed on 22 March 2022), and checked and refined using BioEdit v. 7.2.6 (Hall, 1999). The ITS rDNA, *tef1-α*, and *tub2* matrices were combined for subsequent phylogenetic analyses, and the combined data matrix contained 2265 characters (545 nucleotides of ITS, 900 nucleotides of *tef1-α*, and 820 nucleotides of *tub2*). Maximum likelihood (ML) analyses were performed with RAxML (Stamatakis, 2006), as implemented in raxmlGUI 1.3 (Silvestro and Michalak, 2012), using the ML + rapid bootstrap setting and the GTRGAMMA substitution model with 1000 bootstrap replicates. The matrix was partitioned for the different gene regions. For evaluation and discussion of bootstrap support, values below 70% were considered low, between 70 and 90% medium/moderate, and above 90% high and 100% maximum. Maximum parsimony (MP) bootstrap analyses were performed with PAUP v. 4.0a169 (Swofford, 2003), with 1000 bootstrap replicates using five rounds of heuristic search replicates with random addition of sequences and subsequent TBR branch swapping (MULTREES option in effect, steepest descent option not in effect, COLLAPSE command set to MINBRLEN, and each replicate limited to 1 million rearrangements) during each bootstrap replicate. All molecular characters were unordered and given equal weight; analyses were performed with gaps treated as missing data; and the COLLAPSE command was set to minbrlen.

5.2.3 Pathogenicity Test

Pathogenicity tests were carried out by artificial inoculations using the isolates AC50 (*N. rosae*) and AC46 (*N. siciliana*). Three potted 1-year-old plants of avocado cv. “Hass” grafted on “Zutano” were inoculated with each isolate. Inoculations were made on the stem

after removing a piece of bark with a sterile scalpel blade, placing a mycelial plug (0.3 cm^2) from a 15-day-old culture of each isolate onto the wound and covering it with Parafilm® (American National Can, Chicago, IL, USA) to prevent desiccation. The same number of plants was inoculated with sterile PDA plugs to serve as control. All the inoculated plants were grown in a growth chamber with a 12 h photoperiod and maintained at 25 ± 1 °C. The inoculated plants were monitored weekly for development of symptoms, and a final assessment was conducted 50 days after the inoculations. Re-isolations were performed as described above to fulfill Koch's postulates.

5.2.4 Data Analysis

Data derived from the effect of temperature on mycelial growth rate assay and the lesion length measurements were analyzed in Statistix 10 (Analytical Software, 2013). For analysis of the effect of temperature on mycelial growth, variances of the two assays were tested for the homogeneity using Levene's test and then combined in one dataset. Data of the mycelial growth were first transformed to radial growth rate (cm day^{-1}) and then a nonlinear regression adjustment of the dataset was applied through the generalized Analytis β model, using the equation described by Moral *et al*. (2012). Optimum growth temperature was also calculated according to the equation provided by the same authors (Moral *et al*., 2012). For pathogenicity test, analysis of variance (ANOVA) of the lesions length was performed and the mean differences were compared with Fisher's protected least significance difference (LSD) test at $\alpha = 0.05$.

5.3 Results

5.3.1 Isolation and Morphological Characterization

The disease was observed in a commercial avocado orchard located in Giarre (Catania province) on young plants (two years old, 2–3 months after transplanting). Symptoms observed in the field included stem lesions, wood discoloration with brownish streaking, bark cracking, and dieback. Necrotic lesions were characterized by a shrinkage of the affected tissues and internally the wood appeared darkened and dry (Figure 15). Internal lesions started more frequently from the grafting point. The rootstock showed no symptoms. Isolations frequently yielded *Neopestalotiopsis*-like fungi. A total of eight single-spore isolates were collected and kept in our fungal collection. The highest growth rate for the isolate AC46 (1.13−1cm day−1) was observed at 25 °C. According to the Analytis β model, the optimal growth temperature resulted at 24.6 °C. After 7 days of incubation, no mycelial growth was observed at 35 °C. Isolate AC50 showed the highest growth rate (1.06 cm day^{-1}) at 25 °C, and the optimal growth temperature resulted at 21.9 °C. All results of the effects of temperature on mycelial growth rate are shown in Figures 16 and 17.

Figure 15 Symptoms caused by *Neopestalotiopsis* spp. on avocado. A, Colony of *N. rosae* isolate AC50 grown on PDA for 7 days; B, colony of *N. siciliana* isolate AC46 grown on PDA for 7 days; C, external lesion; D, shrinkage of the necrotic tissue; E, external lesion with bark cracking; F- G, wood discoloration and brownish streaking.

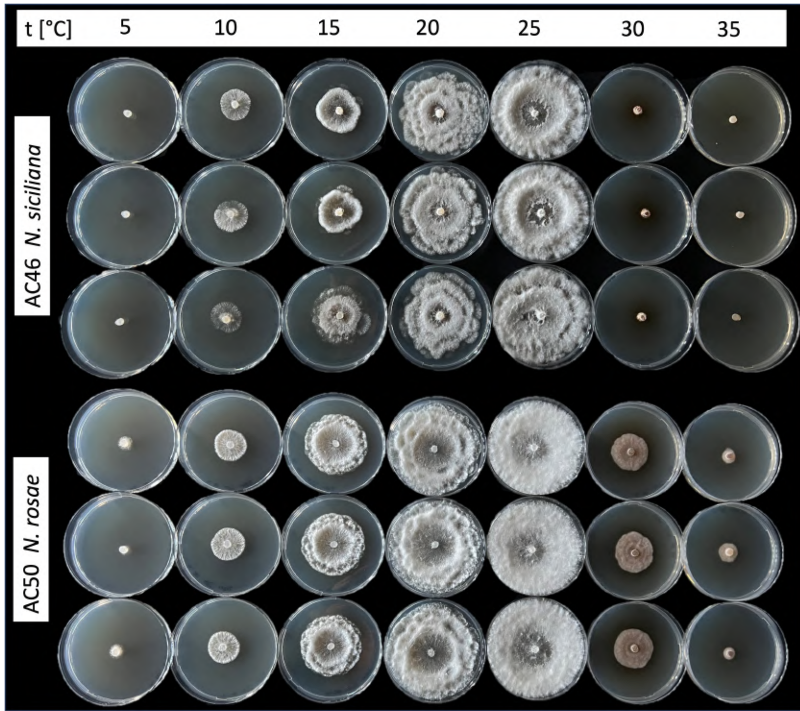

Figure 16 Effect of temperature on mycelial growth rate of two *Neopestalotiopsis* spp. isolated from avocado after 7 days of incubation.

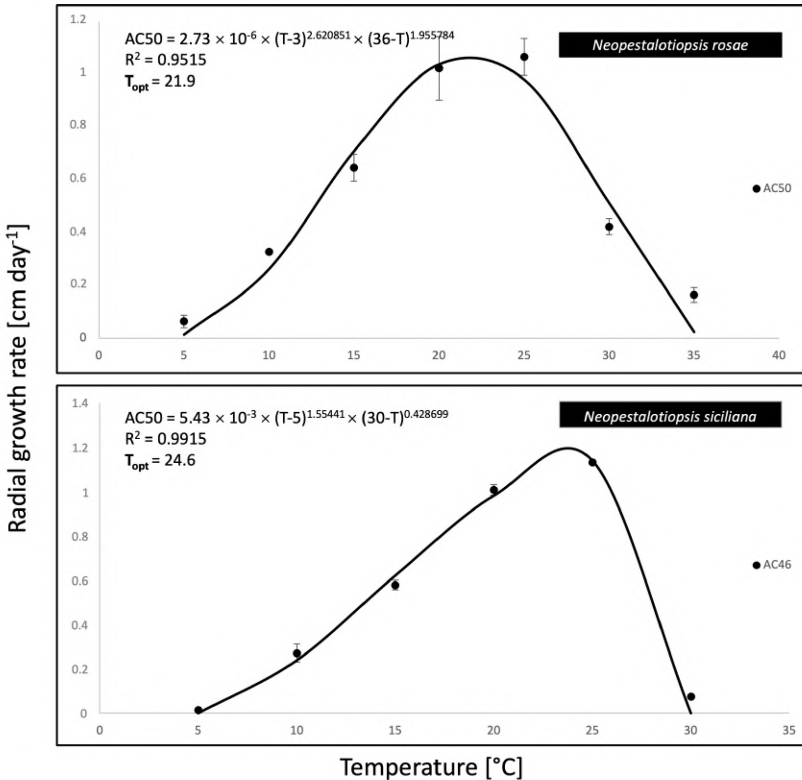

Figure 17 Effect of temperature on mycelial growth rate of two *Neopestalotiopsis* spp. isolated from avocado. The averages of radial growth rate and temperatures were adjusted to a nonlinear regression curve through the Analytis β model. Data points are the means of two independent experiments of three replicated Petri dishes. Vertical bars are the standard error of the means.

5.3.2 Phylogenetic Analysis

PCR amplification of the ITS, *tef1-α*, and tub2 generated 549, 549–550, and 957 bp fragments, respectively. Of the 2261 characters included in the phylogenetic analyses, 334 were parsimony

informative (58 from the ITS, 153 from *TEF1*, and 123 from *tub2*). The best ML tree (−lnL = 8699.596) revealed by RAxML is shown as a phylogram in Figure 18. While backbone support of deeper nodes was mostly absent, several terminal nodes received medium to high support. Of the four *Neopestalotiopsis* isolates of the current study, one was placed within the *N. rosae* clade, while the other three isolates were contained within a moderately supported clade together with four unnamed Japanese isolates from *Eriobotrya japonica*. The latter clade was further subdivided into two subclades: a highly supported subclade containing the three isolates of the present study and one isolate from *Eriobotrya japonica*, and a second, moderately supported subclade containing the residual isolates from *Eriobotrya japonica*.

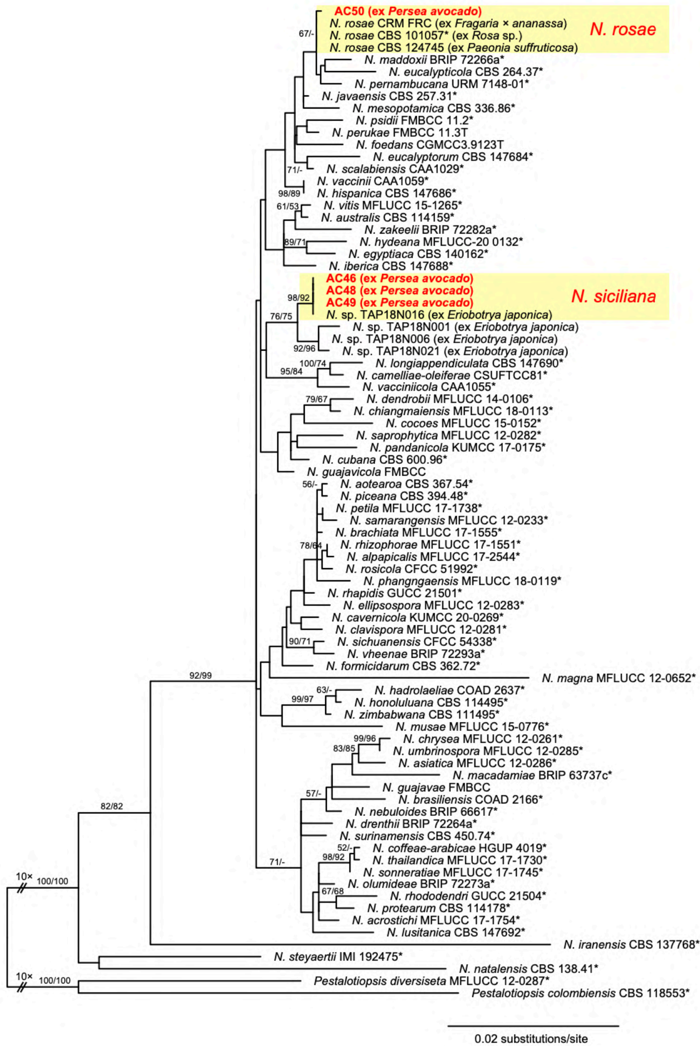

Figure 18 Phylogram of the best ML tree (−lnL = 8699.596) revealed by RAxML from an analysis of the combined ITS-TEF1-tub2 matrix of *Neopestalotiopsis*, showing the phylogenetic position of the isolates obtained from diseased avocado stem tissue (bold red). Strains marked by an asterisk (*) represent ex-type strains. ML

and MP bootstrap support above 50% are given above or below the branches. The broken branches were scaled to one tenth.

5.3.3 Pathogenicity Test

The results of the pathogenicity test showed that both species of *Neopestalotiopsis* identified in this study were pathogenic to avocado and produced the same symptoms similar to those observed in the field. All inoculated trees showed severe external and internal wood discoloration. Controls did not show any symptoms (Figure 19). For both species, the presence of acervuli on the inoculated wounds was observed. The mean lesion lengths of *N. rosae* (7.76 cm) and *N. siciliana* (6.65 cm) were significantly different from the control (0.6 cm) ($p < 0.05$), but not significantly different between them (Figure 20). Re-isolations showed the presence of colonies with the same morphological characteristics as the inoculated species, so Koch's postulates were fulfilled.

Figure 19 Results of pathogenicity test after 50 days. A- B, External and internal lesions caused by *Neopestalotiopsis rosae*; C- D, external and internal lesions caused by *N. siciliana*; E, control. Scale bar = 2 cm.

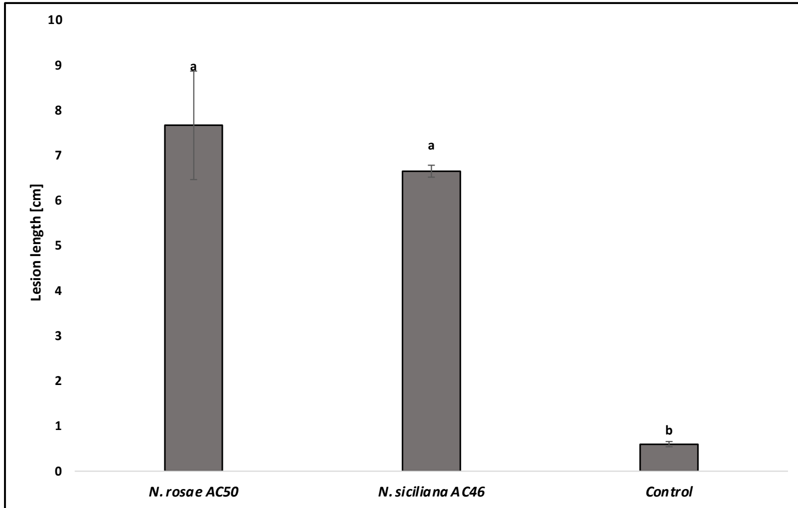

Figure 20 Comparisons of average lesion length (cm) resulting from pathogenicity tests among *Neopestalotiopsis rosae* and *N. siciliana* on potted plants. Columns are the means of 6 inoculation points (2 per plants) for each fungal species. Control consisted of 6 inoculation points. Vertical bars represent the standard error of the means. Bars topped with different letters indicate treatments that were significantly different according to Fisher's protected LSD test (α = 0.05).

5.3.4 Morphological Description of *Neopestalotiopsis rosae* Isolates from Avocado

Neopestalotiopsis rosae **Maharachch., K.D. Hyde and Crous**, in Maharachchikumbura, Hyde, Groenewald, Xu and Crous, *Stud. Mycol*. *79*: 147 (2014) (Figure 21).

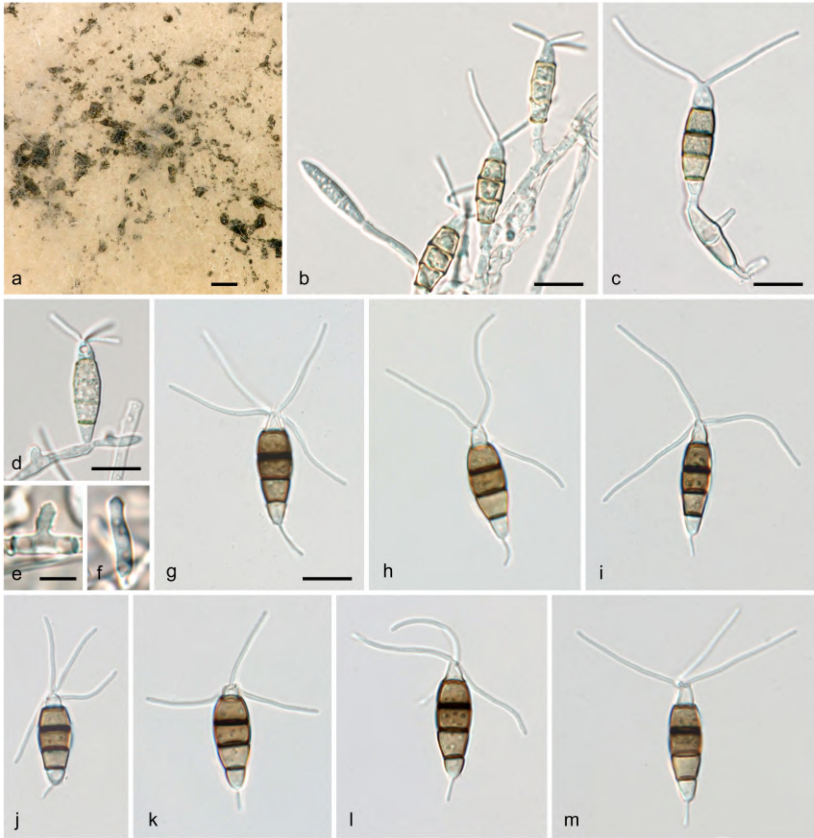

Figure 21 *Neopestalotiopsis rosae* (strain AC50). A, PDA culture with sporodochial conidiomata and black conidial masses; B–D, conidiogenous cells giving rise to conidia; E-F, holoblastic-annelidic conidiogenous cells; G–M, conidia. All in tap water. Scale bars: A = 200 µm; B–D, G–M, = 10 µm, E, F = 5 µm.

Description—sexual morph unknown. Asexual morph: conidiomata on natural substrate acervular, in culture on PDA sporodochial; solitary, pulvinate, black, 30–100 (–150) µm diam., and exuding black conidial masses. Conidiophores indistinct and usually reduced to conidiogenous cells. Conidiogenous cells 1–33 × 1–3.7 µm, discrete,

either short-cylindrical, sitting laterally on hyphae, or cylindrical, ampulliform to lageniform, hyaline, smooth, thin-walled, simple, holoblastic-annelidic, proliferating one to two times percurrently, with collarette present and not flared. Conidia (20–)22–24(–25) × (6.2–)6–8.7 (–15.2) µm, l/*w* = (1.6–)2.9–3.6(–3.9) (*n* = 40), fusoid, straight or slightly curved, four-septate, smooth, slightly constricted at the septa; the basal cell obconic with a truncate base, thin- walled, hyaline or pale brown, and (3.3–)3.8–4.8(–5.4) µm long; three median cells trapezoid or subcylindrical, (8–)14–17(–17) µm long, smooth-walled, versicolored, with septa darker than the rest of the cell; the second cell from the base pale brown and (3.8–)4.6–5.5(–6.1) µm long; the third cell s medium brown and (4–)4.5–5.1(–5.7) µm long; the fourth cell medium brown and (4.4–)5–5.6(–6.1) µm long, septum between the third and fourth cell more distinct, broader, and darker brown than the other septa; the apical cell conic with the subacute apex thin-walled, smooth, hyaline, (2.8–)3.4–4.4(–4.8) µm long, with two to four apical appendages (mostly three) arising from the apical crest; apical appendages unbranched, tubular, centric, and straight or slightly bent, (15–)19–28(–33) µm long, and (0.8–)0.9–1.1 (–1.3) µm wide (*n* = 60); basal appendage single, filiform, unbranched, centric or eccentric, (3–)3.5–5.8(–8.1) µm long and 0.5–0.9 µm wide (*n* = 85).

Culture characteristics. The colony on PDA attaining 90 mm diameter after 7 days at 21.9 °C, yellowish, with a fluffy whitish aerial mycelium, secreting a yellowish pigment in the culture medium, with isolated conidiomata scattered on the aerial mycelium (Figure 22A). The reverse is pale yellowish brown (Figure 22B).

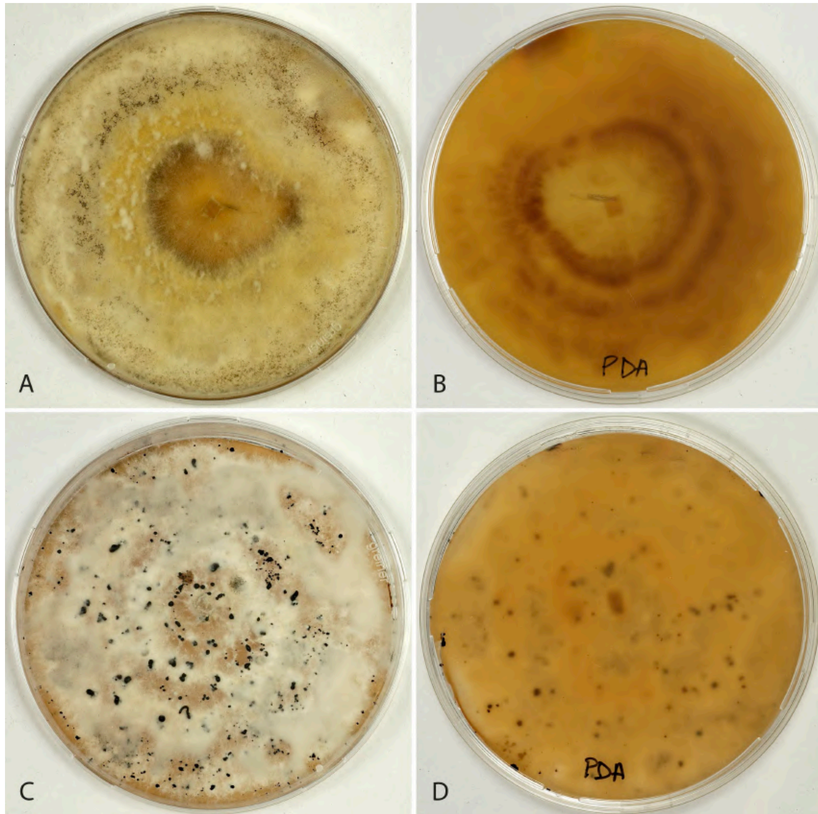

Figure 22 Cultures of *Neopestalotiopsis* spp. from avocado on PDA after 4 weeks. A-B, *N. rosae* isolate AC50 from top (A) and reverse (B); C-D, *N. siciliana* isolate AC46 from top (C) and reverse (D).

Habitat. On stems of *Persea americana* Mill.
Distribution. Sicily, Italy.
Specimens examined. ITALY, Sicily, Catania Province, Giarre, 15 December 2020, Alberto Fiorenza (WU-MYC 0045984; culture AC50 = CBS 149120).
Notes. Our strain AC50 has identical ITS and *TEF1* and highly similar *tub2* (99.8%; 1 nt difference) sequences to the type strain of *N. rosae*

(CBS 101057). *Neopestalotiopsis rosae* has been recorded as a pathogen of various fruit crops, e.g., blueberry (*Vaccinium corymbosum*; (Rodríguez-Gálvez *et al*., 2020; Santos *et al*., 2022), pomegranate (*Punica granatum)*; (Xavier *et al*., 2020)), and in particular strawberry (*Fragaria* × *ananassa*), on which it was reported to cause severe disease outbreaks around the world in recent years (e.g., Australia (Baggio *et al*., 2021), China (Sun *et al*., 2021), Mexico (Rebollar-Alviter *et al*., 2020), Taiwan (Wu *et al*., 2021), and the USA (Baggio *et al*., 2021)). In the protologue of *N. rosae*, it was characterized by three to five tubular apical appendages not arising from the apical crest but at different regions in the upper half of the apical cell. This does not agree with our observations, as in our strain, the apical appendages arise from the apical crest. However, the descriptions and illustrations of the other reports of *N. rosae* cited above agree well with our strain, as do the molecular data.

5.4 Taxonomy

Neopestalotiopsis coffeae-arabicae (Yu Song, K. Geng, K.D. Hyde and Yong Wang bis) Voglmayr, comb. nov. MycoBank No.: MB 844083.

Basionym: *Pestalotiopsis coffeae-arabicae* Yu Song, K. Geng, K.D. Hyde and Yong Wang bis, in Song, Geng, Zhang, Hyde, Zhao, Wei, Kang and Wang, Phytotaxa 126(1): 26 (2013) Notes: This species is clearly a member of *Neopestalotiopsis* according to the results of phylogenetic analyses (Figure 18). Although it was listed as *N. coffeae-arabicae* in various phylogenies (Diogo *et al*., 2021; Liu *et al*., 2021; Norphanphoun *et al*., 2019, Rodríguez-Gálvez *et al*., 2020; Santos *et al*., 2022; Senanayake *et al*., 2020), this combination is neither present in the Index of Fungi nor Mycobank, and could also not be traced in the literature, indicating that it has not been validly

published, which is therefore performed here.

Neopestalotiopsis siciliana Voglmayr, Fiorenza and Aiello, sp. nov.—MycoBank MB 844082; (Figure 23).

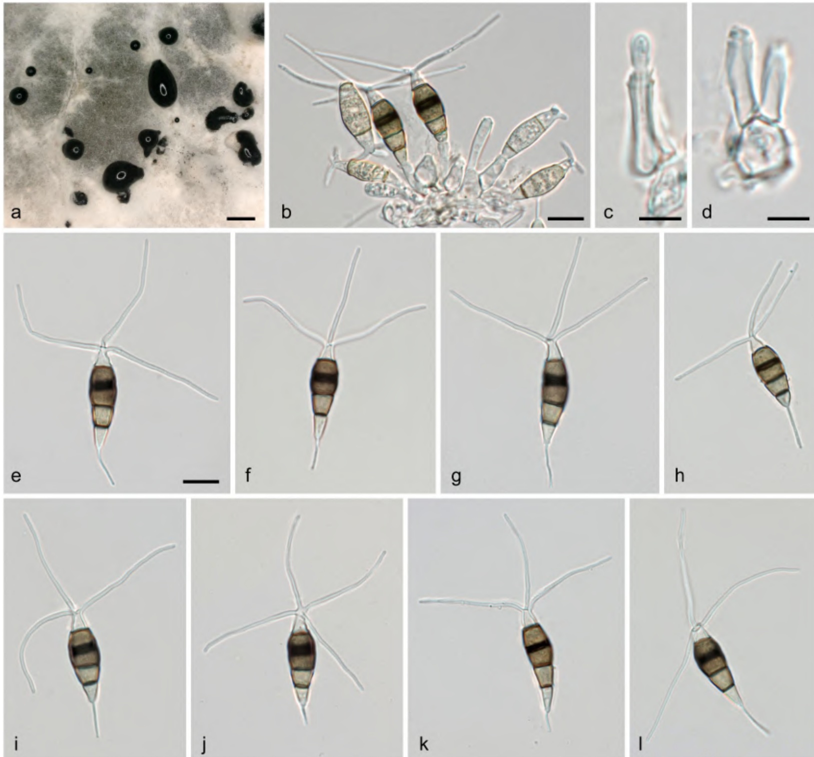

Figure 23 *Neopestalotiopsis siciliana* A–G, I–L, strain AC46, holotype; H, strain AC48). A, PDA culture with sporodochial conidiomata and drops of black conidial masses; B, conidiogenous cells giving rise to conidia; C-D, holoblastic-annelidic conidiogenous cells; E–L, conidia. All in tap water. Scale bars: A = 1 mm; B, E–L = 10 µm, C- D = 5 µm.

Etymology. Named after the region where it was found (Sicily).

Holotype. ITALY, Sicily, Catania Province, Giarre, on stems of *Persea americana*, 15 December 2020, Alberto Fiorenza (WU-MYC 0045982; culture AC46 = CBS 149117).

Description—Sexual morph unknown. Asexual morph: Conidiomata on natural substrate acervular, in culture on PDA sporodochial, solitary, pulvinate, black, and (100–)300–2000(–2800) µm diam., exuding black, globose, and glistening conidial masses. Conidiophores indistinct, usually reduced to conidiogenous cells. Conidiogenous cells 7.7–15.2 × 2.8–6.7 µm, discrete, cylindrical, ampulliform to lageniform, hyaline, smooth, thin-walled, simple, holoblastic-annelidic, and proliferating one to two times percurrently, with collarette present and not flared. Conidia (20–)23–27(–32) × (6–)6.8–7.9(–8.8) µm, l/*w* = (2.8–)3.1–3.8(–4.9) (*n* = 102), fusoid, straight or slightly curved, four-septate, smooth, and slightly constricted at the septa; the basal cell obconic with a truncate base, thin- walled, hyaline or pale brown, (3–)4.3–6(–7.2) µm long; three median cells trapezoid or subcylindrical, (12–)14–17(–23) µm long, smooth-walled, versicolored, with septa darker than the rest of cell; the second cell from the base pale brown and (3.8–)4.5–5.4(–6.4) µm long; the third cell medium brown and (4.1–)4.5–5.5(–7) µm long; the fourth cell medium brown and (3.9–)4.6–5.7(–6.5) µm long; with septum between the third and fourth cell more distinct, broader, and darker brown than the other septa; the apical cell conic with a subacute apex, thin-walled, smooth, hyaline, (3.1–)4.1–5.3(–7) µm long, and with two to four apical appendages (mostly three) arising from the apical crest; apical appendages unbranched, tubular, centric, and straight or slightly bent, (19–)24–34(–38) µm long and (0.9–)1.1–1.5(–1.7) µm wide (*n* = 105); basal appendage single, filiform, unbranched, centric, (2.8–)4.6–9.3(–15.3) µm long, and (0.5–)0.7–0.9(–1.1) µm wide (*n* = 85).

Culture characteristics. Colony on PDA attaining 90 mm diameter after 7 days at 24.6°C, dirty white, with fluffy white aerial mycelium,

conidiomata scattered, isolated (Figure 22C). Reverse pale buff (Figure 22D).
Habitat. On stems of *Persea americana* Mill.
Distribution. Sicily, Italy.
Specimens examined. ITALY, Sicily, Catania Province, Giarre, 15 December 2020, collector Alberto Fiorenza (WU-MYC 0045983; culture AC48 = CBS CBS 149118); Giarre, 15 December 2020, collector Alberto Fiorenza (culture AC49 = CBS 149119).
Notes. The phylogenetic analyses revealed a highly supported, distinct phylogenetic position within *Neopestalotiopsis*, confirming its status as a new species. Remarkably, it clusters with four unnamed *Neopestalotiopsis* accessions isolated from the leaves and fruits of *Eriobotrya japonica* in Japan (Nozawa *et al*., 2020). While one of these strains has sequences identical to our isolates, with which it clustered within a highly supported subclade, the three other strains from *Eriobotrya* form a sister clade to the former (Figure 18). As there are some molecular differences between these two subclades, it is yet unclear whether one or two species are involved; considering the uncertainties in species circumscription in the genus and the lack of morphological data, we here maintain these isolates as *Neopestalotiopsis* sp. As usually observed in *Neopestalotiopsis*, it is impossible to identify *N. siciliana* by conidial morphology alone, and sequence data are necessary for reliable species identification.

5.5 Discussion

The results of this study confirm the presence of *Neopestalotiopsis* species causing disease on young avocado plants in southern Italy. The fungal species obtained from symptomatic tissues were identified based on the morphological characteristics and molecular phylogenetic analyses of the ITS, *tef1-α*, and *tub2* gene

regions. The phylogenetic analyses showed that two species are involved in avocado stem and wood lesions, resulting in the dieback of the plants. Of the four isolates sequenced, one was identified as *N. rosae*, while another three isolates formed a clade distinct from the other described *Neopestalotiopsis* species, which is therefore here described as a new species, *N. siciliana*. Remarkably, these three isolates were phylogenetically close to four unnamed *Neopestalotiopsis* strains isolated from *Eriobotrya japonica* in Japan (Nozawa *et al*., 2020); one even had identical sequences to our isolates. This demonstrates that *N. siciliana* is widespread and has a wider host range. There are a few reports from avocados attributable to the genus *Neopestalotiopsis*, for which ITS sequences are available. Valencia *et al*. (2011) recorded *N. clavispora* (as *Pestalotiopsis clavispora*; ITS sequence HQ659767) as a causal agent of post-harvest stem-end rot in Chile, while Shetty *et al*. (2016) identified one of their endophytic isolates from organically grown avocado trees in South Florida as *Neopestalotiopsis foedans* (ITS sequence KU593530). A sequence comparison of the ITS sequences with our matrix showed that the ITS sequence HQ659767 was identical and that KU593530 was almost identical (one gap difference) to our isolate AC50 representing *N. rosae*. While this could indicate that *N. rosae* is regularly found on avocado, it needs to be noted that the ITS alone is not suitable for species identification, as several species (e.g., *N. hispanica*, *N. longiappendiculata*, *N. mesopotamica*, *N. scalabiensis*, and *N. vaccinii*) have ITS sequences identical to those of *N. rosae*. Therefore, the species identity of these avocado isolates remains uncertain in the lack of *tef1-α* and *tub2* sequences.

Pestalotiopsis sensu lato was recently revised by Maharachchikumbura *et al*. (2014b) and segregated into three distinct genera, viz. *Pestalotiopsis*, *Pseudopestalotiopsis*, and *Neopestalotiopsis*. During our field surveys, we often encountered young plants of avocado showing stunted growth. Monitoring the

plants after the transplant from the nurseries to the open field, it was noticed that some of these were not able to survive. Deeper observations of the internal tissues revealed frequent necrosis and cankers at the grafting point. Likely, propagation processes represent relevant infection courts for pestalotioid fungi. Most of the avocado plants transplanted in Sicily derive from Spanish nurseries where the propagation steps are performed. It is not unusual that symptoms such as stunting, shoot blight, and cankers observed by the growers after the first years from the transplant in the open field are the results of previous infections that had occurred in the nurseries, especially during the grafting. In fact, wounds and injuries are crucial for penetration of the host tissue and subsequent development of the infection, especially for pestalotioid species (Espinoza *et al*., 2008). Our observations are indeed in accordance with other reports. In China, 30% of symptomatic avocado plants in a nursery plantation showed the presence of *Pestalotiopsis longiseta* (Lin *et al*., 2018) and other authors also reported the presence of *Pestalotiopsis* spp. at the graft union in different hosts (Cardoso *et al*., 2002; Gibson and Howland, 1969; Rego *et al*., 2006). Species of these genera are widely distributed in tropical and temperature areas. This group of fungi is commonly found as endophytes and plant pathogens on different hosts, causing stem-end rot, stem and leaf blight, trunk disease, and cankers (Ismail *et al*., 2013; Maharachchikumbura *et al*., 2014b). Previous investigation conducted in Sicily already reported the presence of pestalotioid fungi, including *Pestalotiopsis clavispora* and *P. uvicola*, causing diseases on tropical, as well as on ornamental, hosts (Ismail *et al*., 2013; Vitale *et al*., 2005). This study represents a new step forward in the insight of this complex and still understudied group of fungi, especially within the genus of *Neopestalotiopsis*, providing new information on the ecology of these two species.

5.6 Conclusions

Two fungal species, *Neopestalotiopsis rosae* and *N. siciliana* sp. nov. are described and illustrated. These fungi were isolated from the stem tissues of diseased young avocado plants in Sicily, Italy. Pathogenicity tests were performed, and Koch's postulates were fulfilled. The result of this study provided new information regarding this still understudied group of phytopathogenic fungi and its wide host range. *Neopestalotiopsis rosae* and *N. siciliana* could be a new threat to the Italian avocado industry, especially in the southern regions where avocado represents an emerging crop. The presence of these species in the internal tissues at the graft union corroborates the fact that the propagation processes represent crucial steps to obtain healthy material. Further investigations are needed in order to ascertain the diffusion and epidemiology of these species in the Sicilian avocado orchards, and to evaluate the effective risk for the industry. Therefore, it will be important to carry out additional studies on the pathogenicity and susceptibility of the different cultivars of avocado in the future. To our knowledge, this is the first report of *N. rosea* and of the fungus here described as *N. siciliana* affecting avocado.

5.7 References

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6. Other research activities: First Report of *Rosellinia necatrix* Causing White Root Rot on Avocado in Italy

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Key words: *Rosellinia necatrix*, white root rot, avocado, morphological and molecular characterization.

Avocado (*Persea americana* Mill.) is a versatile crop that could replace lemon, especially along the east coast of Sicily Island, and it is suitable for cultivation in other temperate areas of southern Italy (Calabria). During November of 2020, a rapid decline, yellowing, and leaf wilting were observed on ~2,000 potted 10- to 12-month-old avocado cultivar Zutano seedlings grown in a farm located in Catania province (eastern Sicily). More than 8% of the seedlings showed disease symptoms including leaf yellowing and general wilting. Dark brown lesions were present at the base of the trunk, with white mycelia extending under the bark (Figure 24). The infected roots were necrotic and covered with a fine layer of white mycelia (Figure 24). Crown and root rotting tissues of five seedlings were surface disinfected for 1 min in 1.5% sodium hypochlorite solution, rinsed in sterile water, and placed on potato dextrose agar (PDA) amended with 100 mg/liter of streptomycin sulfate and incubated at 25°C for 7 days. Fungal cultures consistently isolated from infected tissues were purified by using the hyphal tip method. All cultures showed pyriform swellings at the base of the septa of the hyper mycelia, which is characteristic of the genus *Rosellinia* De Notaris. Young mycelia were initially white and cottony but turned brown or black with age. Conidia were solitary, one-celled, aseptate, hyaline, and elliptical. Mean of a total of 50 conidia was 3 to 5 μm long × 2.5 to 3 μm wide. For five representative isolates, the internal transcribed spacer (ITS) region of rDNA and actin were amplified with primers ITS5/ITS4 (White *et al*. 1990) and ACT-512F/ACT-783R (Carbone and Kohn, 1999), respectively. The sequences of the isolates were deposited in GenBank (accession nos. MW582604, MW582605, MW582606, MW582607, and MW582608 for ITS and MW620212, MW620213, MW620214, MW620215, and MW620216 for actin). Nucleotide BLAST analysis showed 100% identity with *Rosellinia necatrix* Prill. (MF611983) for ITS, and the same result was obtained with actin (MG273315). Pathogenicity tests were performed on 12 potted, healthy, 1-year-old seedlings of avocado

cultivar Zutano. A piece of crown bark was removed with a 6-mm-diameter cork borer, and a mycelial plug of the same size was placed in the wound. Twelve control plants were inoculated with a sterile PDA plug. All avocado plants were kept in a growth chamber with a 12-h photoperiod at 25 ± 1°C and watered weekly. Pathogenicity tests led to identical symptoms to those observed in the field. After 5 weeks, all inoculated plants showed yellowing and wilting of the leaves. After 10 weeks all plants were dead. The presence of white mycelia was noticed in the soil around the crown roots (Figure 24). Control plants remained symptomless. *R. necatrix* were successfully reisolated from all symptomatic tissues and identified as previously described. White root rot is a serious threat in the Mediterranean area (Pliego *et al*., 2009), and it is considered the main cause of endemic root rot in Spain (López-Herrera *et al*., 1998). In southern Italy, previous studies revealed the importance of other fungal diseases on the avocado crop (Aiello *et al*., 2020; Guarnaccia *et al*., 2016, 2018; Vitale *et* al., 2012). Moreover, the presence of *R. necatrix* could be a relevant limiting factor to the Sicilian avocado industry. To our knowledge, this is the first report of white root rot on avocado in Italy.

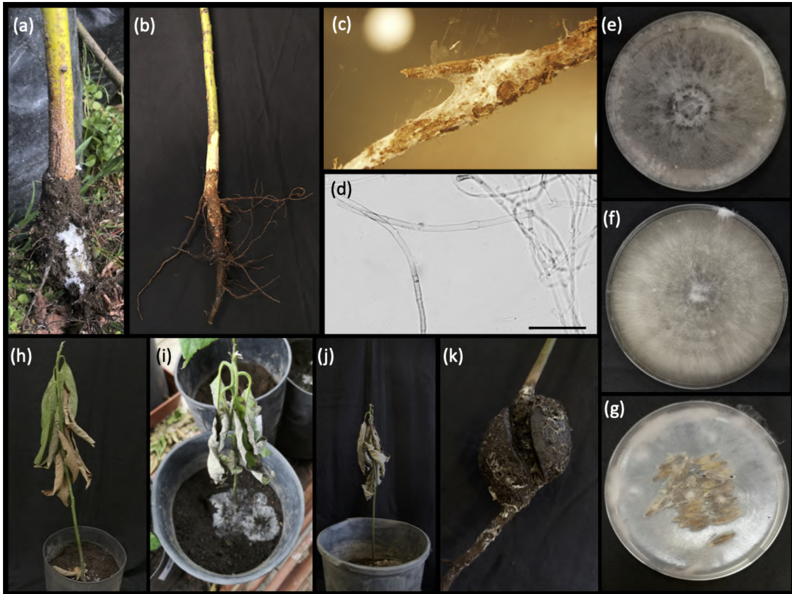

Figure 24. a-b, Symptoms caused by *Rosellinia necatrix* on crowns and roots of avocado seedlings; c, root with fine white mycelia layer; d, typical pear-shaped swelling in the septa union of mycelia, scale bar: 50 µm; e, colony on PDA (14-day-old); f, colony on OA (14-day-old); g, colony on PDA amended with oat kernels (14- day-old); h-k, symptoms on inoculated seedlings.

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7. Other research activities: *Fusarium nirenbergiae* (*Fusarium oxysporum* Species Complex) Causing the Wilting of Passion Fruit in Italy

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Key words: wilt, passion fruit, *Fusarium oxysporum* species complex.

7.1 Introduction

In recent years, tropical fruit production increased worldwide due to the increasing demand of global markets and more efficient transportation and storage techniques (Ding, 2017; Underhill, 2003). Most of the tropical fruit is destined for fresh consumption or industrial transformation. Among these, passion fruit (*Passiflora edulis* Sims.) is one of the most exported and consumed fruit commodities. It originated in tropical and subtropical America (Cervi, 2006), and it is now extensively cultivated worldwide, including Australia, New Zealand, India, Africa, and South America (Manicom *et al*., 2003; Vanderplank, 1996). Passion fruit is mainly cultivated for its edible fruit but secondarily also for its attractive flowers on ornamental evergreen vines.

In Italy, the cultivation of *P. edulis* (also known as purple passion fruit) as a fruit crop in some regions characterized by a Mediterranean climate (e.g., Sicily and Calabria) is gaining growing interest by local farmers, and it is carried out under greenhouse and, to a lower extent, open field conditions. Indeed, although the crop is well adapted to a wide rainfall range (1000–2500 mm for crop season), minimum temperatures below 5 °C should be avoided because they seriously compromise the plant growth and nutrient uptake (Das *et al*., 2013; NDA-ARC, 1999; Rao *et al*., 2013). In this regard, it should be noted that a process of reconversion of protected tomato and vegetable crops into tropical fruit plantations is currently taking place in southern Italy and Sicily. Unfortunately, this species is affected by many diseases during its different growth stages, and this reduces the yield and the farmers' income (Fischer *et al*., 2005). One of the most widely reported fungal pathogens affecting passion fruit is *Fusarium oxysporum* f. sp. *passiflorae*, which causes the Fusarium wilt. It was first reported in Australia (McKnight, 1951) but is nowadays spread

worldwide (Garcia *at al*., 2019; Liberato et *al*., 2001; Rooney-Latham *et al*., 2011). Among *Fusarium* diseases, *Neocosmospora solani* (=*Fusarium solani*) is responsible for the basal stem rot (Fischer *et al*., 2008; Li *et al*., 1993; Ploetz, 1991). According to Viana and Costa (2003), the species *F. oxysporum* f. sp. *passiflorae* and *N. solani* are the most damaging ones to passion fruit crops. Minor diseases have been reported on passion fruit, such as the damping-off of seedlings and collar and root rot in adult plants caused by *Rhizoctonia solani* (Bezerra and Oliveira, 1984) and collar rot caused by *Phytophthora* spp. (Young, 1970). During a recent survey performed in Sicily, young passion fruit plants showing symptoms of general yellowing and wilting were observed in some of the most representative production areas. Given the increasing interest of local growers in expanding passion fruit cultivation, the aim of this study was to characterize the fungal species associated with those symptoms and test their pathogenicity, in order to better understand the syndrome's aetiology.

7.2 Materials and methods

7.2.1 Field Survey, Isolations and Morphological Characterization

In July of 2020, 50 two-year-old 'purple' passion fruit plants cultivated in a greenhouse in the Syracuse province (Sicily, Italy) appeared stunted, defoliated and severely wilted. Diseased vascular tissues (0.5 cm^2) were surface-disinfected for 1 min in a 1.2% sodium hypochlorite (NaOCl) solution, rinsed in sterile water, placed on a potato dextrose agar (PDA, Lickson, Vicari, Italy) amended with 0.1 g/L of streptomycin sulphate (Sigma- Aldrich, St. Louis, MO, USA), to prevent bacterial growth, and then incubated at 25 ± 1 °C until fungal colonies were observed. Single-spore isolates were obtained

from pure cultures grown on APDA. To induce sporulation, five representative single-spore isolates (named Di3A-Pef1, Di3A-Pef2, Di3A-Pef3, Di3A-Pef4 and Di3A-Pef5) were selected and transferred on a synthetic nutrient-poor agar (SNA) (Nirenberg, 1891), Oatmeal Agar (OA, Difco, Detroit, MI, USA) and PDA for morphological characterization. A total of 50 macro- and micro-conidia were measured (length and width size) using a fluorescence microscope (Olympus-BX61) coupled to an Olympus DP70 digital camera; images and measurements were captured using the software analySIS Image Processing. Conidia sizes are reported as the minimum and maximum in parentheses, and the average is reported with the standard deviation.

7.2.2 Molecular Characterization and Phylogeny

Genomic DNA of the selected isolates (Di3A-Pef 1-2-3-4-5) was extracted using the Gentra Puregene Yeast/Bact kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The internal transcribed spacer of the ribosomal DNA (rDNA-ITS), partial translation elongation factor alpha gene (*tef1*-α) and RNA polymerase II gene (*rpb2*) were targeted for PCR amplification and sequencing. The primers used for these regions were: ITS5 and ITS4 for ITS (White *et al*., 1990), EF1-728F and EF1-986R for *tef1*-α (Carbone and Kohn, 1999) and 5f2 and 7cr for *rpb2* (Liu *et al*., 1999). The PCR products were purified and sequenced in both directions by Macrogen Inc. (Seoul, Korea). The sequences were edited using MEGAX: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar *et al*., 2018), manual adjustments of alignments were made when necessary and submitted to GenBank. Moreover, the sequences were blasted in the NCBIs GenBank nucleotide database and on the Fusarium MLST database of the Westerdijk Fungal

Biodiversity Institute (http://www.westerdijkinstitute.nl/fusarium/, accessed on 21 May 2021). For comparison, 44 additional sequences were selected according to the recent literature (Lombard *et al*., 2019) (Table 5). The phylogenetic analysis was based on the Maximum Parsimony (MP). The MP analysis was done using PAUP v. 4.0a165 (Swofford, 2002). Phylogenetic relationships were estimated by heuristic searches with 100 random addition sequences. A tree bisection-reconnection was used, with the branch swapping option set to 'best trees' only, with all characters weighted equally and alignment gaps treated as the fifth state. The tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated for parsimony and the bootstrap analyses were based on 1000 replicates (Hillis and Bull, 1993). *Fusarium foetens* (CBS 120665) and *F. udum* (CBS 12881) served as outgroups.

Table 5 Characteristics of *Fusarium* isolates included in the phylogenetic analysis.

Species	Culture accession	Host/substrati	Special form	Origin	GeneBank accession	
					rpb2	***tef1-α***
Fusarium callistephi	CBS 187.53	*Callistephus chinensis*	*callistephi*	The Netherlands	MH484875	MH484966
F. carminascens	CBS 144739	*Zea mays*		South Africa	MH484934	MH485025
F. cugenengense	CBS 620.72	*Crocus* sp.	*gladioli*	Germany	MH484879	MH484970
F. cugenengense	CBS 130304	*Gossypium barbadense*	*vasinfectum*	China	MH484921	MH485012
F.curvatum	CBS 247.61	*Matthiola incana*	*matthiolae*	Germany	MH484876	MH484967
F.curvatum	CBS 238.94	*Beaucarnia* sp.	*meniscoideum*	The Netherlands	MH484893	MH484984
F. duoseptatum	CBS 102026	*M. sapientum*	*cubense*	Malaysia	MH484896	MH484987
F. elaeidis	CBS 217.49	*Elaeis* sp.	*elaeidis*	Zaire	MH484870	MH484961
F. fabacearum	CBS 144742	*Zea mays*		South Africa	MH484938	MH485029
F. foetens	CBS 120665	*Nicotiana tabacum*		Iran	MH484918	MH485009
F. glycines	CBS 144746	*Glycine max*		South Africa	MH484942	MH485033
F. glycines	CBS 20089	*Ocimum basilicum*	*basilici*	Italy	MH484888	MH484979
F. glycines	CBS 17633	*Linum usitatissium*	*lini*	Unknow	MH484868	MH484959
F. glycines	CBS 21449	Unknow		Argentina	MH484869	MH484960
F. gossypinum	CBS 116611	*Gossypium hirsutum*	*vasinfectum*	Ivory Coast	MH484907	MH484998
F. hoodiae	CBS 132474	*Hoodia gordonii*	*hoodiae*	South Africa	MH484929	MH485020
F. languescens	CBS 41390	*Solanum lycopersicum*	*lycopersici*	Israel	MH484890	MH484981
F. languescens	CBS 119796	*Zea mays*		South Africa	MH484917	MH485008

F. languescens	CBS 30291	*Solanum lycopersicum*	*lycopersici*	The Netherlands	MH484892	MH484983
F. languescens	CBS 646.78	*Solanum lycopersicum*	*lycopersici*	Morocco	MH484881	MH484972
F. nirembergiae	CBS 744.79	*Passiflora edulis*	*passiflorae*	Brazil	MH484882	MH484973
F. nirembergiae	CBS 115424	*A. betulina*		South Africa	MH484906	MH484997
F. nirembergiae	CBS 12924	*Secale cereale*		Unknow	MH484864	MH484955
F. nirembergiae	CBS 12781	*Chrysanthemum* sp.	*chrysanthemi*	USA	MH484883	MH484974
F. nirembergiae	CBS 130303	*Solanum lycopersicum*	*radicis-lycopersici*	USA	MH484923	MH485014
F. nirembergiae	CBS 115417	*A. betulina*		South Africa	MH484903	MH484994
F. nirembergiae	CBS 19687	*Bouvardia longiflora*	*bouvardiae*	Italy	MH484886	MH484977
F. nirembergiae	CBS 123062	Tulip roots		USA	MH484919	MH485010
F. nirembergiae	CBS 18132	*S. tuberosum*		USA	MH484867	MH484958
F. nirembergiae	CBS 75868	*S. lycopersicum*	*lycopersici*	The Netherlands	MH484877	MH484968
F. nirembergiae	CBS 840.88	*Dianthus caryophyllus*	*dianthi*	The Netherlands	MH484887	MH484978
F. oxysporum	CBS 221.49	*Camellia sinensis*	*medicaginis*	South East Asia	MH484872	MH484963
F. oxysporum	CPC 25822	*Protea* sp.		South Africa	MH484943	MH485034
F. oxysporum	CBS 144134	*S. tuberosum*		Germany	MH484953	MH485044
F. pharetrum	CBS 144751	*A. dichotomum*		South Africa	MH484952	MH485043
F. pharetrum	CBS 144750	*Aliodendron dichotomum*		South Africa	MH484951	MH485042
F. trachichlamydosporum	CBS 102028	*M. sapientum*	*cubense*	Malaysia	MH484897	MH484988
F. triseptatum	CBS 258.50	*Ipomea batatas*	*batatas*	USA	MH484873	MH484964

F. triseptatum	CBS 116619	*G. hirsutum*	*vasinfectum*	Ivory Coast	MH484910	MH485001
F. udum	CBS 177.31	*Digitaria ariantha*		South Africa	MH484866	MH484957
Fusarium sp.	CBS 12881	*Chrysanthemum* sp.	*chrysanthemi*	USA	MH484884	MH484975
Fusarium sp.	CBS 130323	Human nail		Australia	MH484927	MH485018
Fusarium sp.	CBS 68089	*Cucumis sativus*	*cucurbitacearum*	The Netherlands	MH484889	MH484980

7.2.3 Pathogenicity Tests

In order to fulfil Koch's postulates, pathogenicity tests were conducted on one-year- old potted cuttings using the mycelial plug technique. In detail, 18 healthy cuttings were inoculated, removing a piece of bark of the crown root with a scalpel blade and applying a mycelial plug (0.3 cm^2), taken from a 14-day-old Di3A-Pef 1 isolate, upside down on the wound and subsequently covered with soil to prevent desiccation. The controls consisted of sterile PDA plugs applied as described above to the same number of healthy young plants. Re-isolation attempts were performed from representative inoculated plants.

7.3 Results

The symptoms observed in the greenhouse consisted of leaf yellowing and wilting (Figure 25a,b), external crown and root rot and wood discoloration moving upward to the canopy (Figure 25c). The disease incidence reached 10% of the cultivated plants. Colonies with white or light purple aerial mycelia and violet pigmentation on the underside of the cultures developed after 14 days on PDA, being firstly identified as *Fusarium* - like. Sporodochial macroconidia with 2 to 5 septa, grown on OA, measured (23.09–) 28.76 ± 3.06 (–35.48) µm × (1.99–) 3.84 ± 0.58 (–4.75) µm (Figure 25f,g). Oval, unicellular microconidia developed on short monophialides, grown on OA, measured (3.1–) 5.17 ± 1.35 (–9.17) µm × (1.3–) 1.98 ± 0.37 (–2.9) µm (Figure 25i).

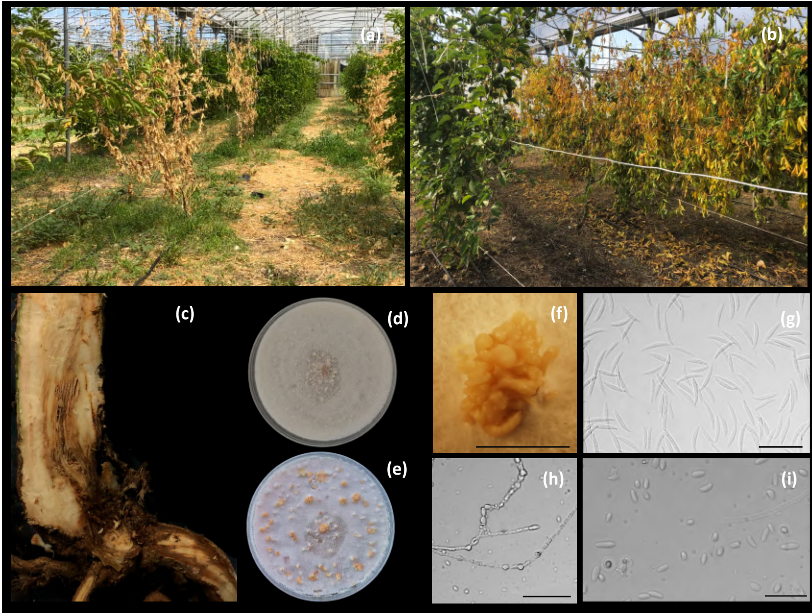

Figure 25 Disease symptoms and *Fusarium nirenbergiae* features: a-b, yellowing and wilting of passion fruit plants in greenhouse; c, vascular discoloration on a collar portion; d-e, *F. nirenbergiae* (Di3A-Pef1 isolate) grown on 7 day-old (up) and 14 day-old (down) OA; f, sporodochia on OA; g, sporodochial conidia (macroconidia); h, chlamydospores on SNA; i, aerial conidia (microconidia). Scale bars, f: 2 mm; g–i: 50 µm.

PCR edit amplicons resulted in 528 bp for the partial ITS region, 287 bp for *tef1*-α and 953 bp for *rpb2*. The sequences were registered in GenBank as follows: MZ398141, MZ398142, MZ398143, MZ398144, MZ398145 for ITS, MZ408109, MZ408110, MZ408111, MZ408112, MZ408113 for *rpb2* and MZ408114, MZ408115, MZ408116, MZ408117, MZ408118 for *tef1*-α. A GenBank BLASTn analysis and a pairwise sequence alignment on the MLST database indicated that all the isolates from passion fruit belonged to the *Fusarium oxysporum* species complex (FOSC). In particular, the

MLST search resulted in high identity values (96–100%) (Acc. number MH582354) for the *tef1*-α gene and 98% (Acc. number MH582140) for the *rbp2* gene with a *F. oxysporum* species complex (FOSC). The MP heuristic search resulted in 83 parsimony-informative characters, while 109 were variable and parsimony-uninformative and 1412 were constant. A maximum of 320 equally most parsimonious trees were retained (Tree length = 249, CI = 0.851, RI = 0.898 and RC = 0.765). The bootstrap support values from the parsimony analysis are shown close to the branch node. The group of representative isolates Di3A-Pef1-5 clustered with the reference strain of *F. nirenbergiae*, as shown in Figure 26, and were clearly separated by the other sequences provided in the study by Lombard *et al*. (2019). The isolates were then identified as *Fusarium nirenbergiae* L. Lombard & Crous.

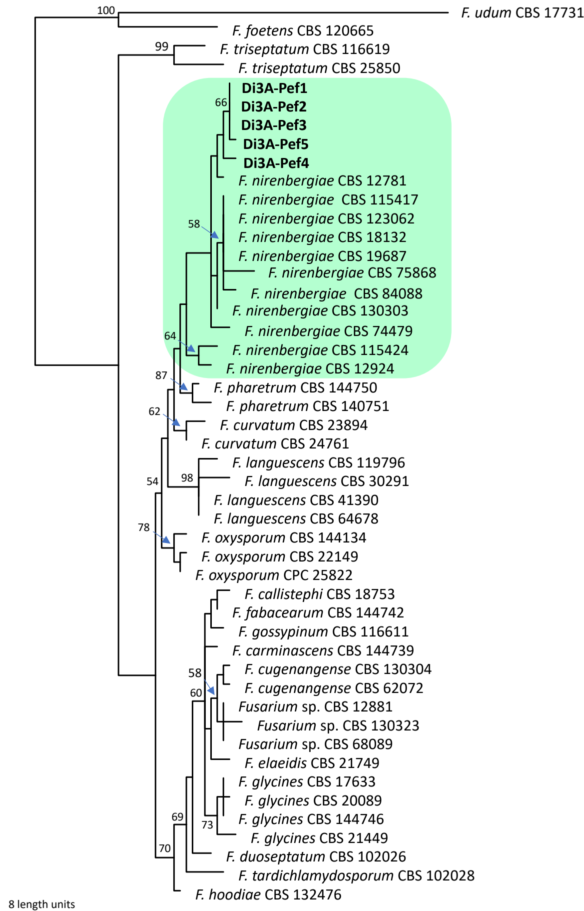

Figure 26 Single most parsimonious phylogenetic tree resulting from the MP analysis of combined *tef1*-α and *rbp2* sequence data. The isolates in bold were sequenced in this study. The numbers represent MP bootstrap values.

The inoculated isolate after five months caused symptoms similar to those observed under greenhouse conditions in all inoculated plants. The symptoms consisted of leaf yellowing and wilting. After 7 months all plants died. A longitudinal section of the inoculated plants reveals

the internal discolorations moving upward to the canopy. The control remains symptomless. From the symptomatic tissues, *F. nirembergiae* was always re-isolated, and it was characterized as previously described.

7.4 Discussion and conclusion

To the best of our knowledge, this paper represents the first report of *F. nirembergiae*, belonging to the FOSC complex, as a causal agent of Fusarium wilt of passion fruit. In this regard, both the morphological characterization and the analysis of the ITS, *tef1*-α and *rbp2* sequences allowed us to correctly allocate a representative number of detected strains within the *F. nirenbergiae* group, being distinctly separated by the other taxa, as recently shown by Lombard *et al*. (2019) and Crous *et al*. (2021). Based on the present findings, *F. nirenbergiae* was strongly grouped in a separated subclade of FOSC, phylogenetically close to *F. curvatum*. Although little information regarding *F. nirembergiae*'s pathogenicity and host range is currently available, except for the study by Zhao *et al*. (2020) on *Acer negundo*, this species (belonging to FOSC) is able to colonize and infect host vascular tissues; for this reason, it is reported worldwide as responsible for Fusarium wilt (Fischer and Rezende, 2008). The first symptoms consist of leaf yellowing and wilt, followed by the plant's collapse. This disease is observed in adult and young plants under favorable conditions for the infection development, such as high temperature and humidity and a high potential inoculum in the soil (Bennett and Davis, 2013; Vanderplank, 1996). Once this fungal pathogen is established in the field, its control is very difficult, since fungicide application does not result in a significant reduction of the disease amount, and the pathogen can persist in the soil for many years in the absence of the host (Fischer and Rezende, 2008). Hence, the

incidence data are very worrying as regards the nature of the fungal pathogen and dissemination ability of *F. nirenbergiae* under greenhouse conditions. If, on the one hand, protected systems could facilitate the cultivation of purple passion fruit, on the other hand they could aggravate the consequences of this phytopathological issue. Indeed, this could represent a future threat for the expansion of this tropical crop, which is replacing protected tomato cultivation in different areas of southern Italy. Therefore, disease management should be focused mainly on preventative and pathogen exclusion measures, avoiding plantation in areas with a severe history of Fusarium wilt infections or selecting healthy propagation material in combination with adequate agronomic practices. Additionally, other sustainable strategies should include the use of resistant cultivars, as recommended by several authors (De Carvalho *et al*., 2021; Silva *et al*., 2013). Comprehensively, the increasing trend of tropical plantations in Italy leads us to focus more on fungal diseases that could represent limiting factors for future production. According to presented data combined with recent findings (Crous *et al*., 2021; Lombard *et al*., 2019), it cannot be excluded that some past reports of *F. oxysporum* f. sp. *passiflorae* could confirm that *F. nirembergiae* is a causal agent of Fusarium wilt. However, further surveys should be performed on *P. edulis* orchards in Italy and worldwide to confirm the new aetiology of the Fusarium wilt of passion fruit and its real diffusion.

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8. Other research activities: Unusual Stylar-End Breakdown and Sour Rot on Key Lime (*Citrus aurantiifolia*) in Pre-Harvest Condition in Italy

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8.1 Introduction

Lime is a hesperidium fruit (*Rutaceae*) classified in three groups: sweet lime (*Citrus limetta*), acid lime, including the "Key" lime (*C. aurantiifolia*), and the Australian finger lime (*C. australasica*) (Mabberley, 2004). Global top producers of lemons/limes (1000 metric tons unit) include Mexico (2870), the European Union (1640) and Argentina (1030) (USDA, 2021). Many pathogen-pest complexes are known as a severe threat for lime production around the world. Regarding fungal diseases, Anthracnose (*Colletotrichum acutatum*), Melanose (*Diaporthe citri*), Scab (*Elsinoe fawcettii*) and Stem-end rot (*Lasiodiplodia theobromae* and *Phomopsis citri*) are the main diseases reported (Al-Sadi *et al*., 2017; Donkersley *et al*., 2018; Rivera-Cabrera *et al*., 2017). Important post-harvest diseases are caused by *Penicillium digitatum* (green mould), *P. italicum* (blue mould) and *Geotrichum candidum* (sour rot) (Al-Sadi *et al*., 2017; Hernández-Montiel *et al*., 2010; Morsy and El-Kader, 1994). No less important than pathological decays, post-harvest disorders such as chilling injury, oil spotting (oleocellosis) and stylar-end breakdown (SEB) represent a significant limiting factor of lime quality (Ferguson and Grafton-Cardwell, 2014; Rivera-Cabrera *et al*., 2017). A survey in a commercial Key lime orchard in Sicily (Italy) cultivated under shade netting revealed an abundant presence of fruit (attached to the trees and harvested as well) showing lesions at the stylar-end. Part of the fruit observed in the field showed water-soaked spots at the stylar-end turning from light to dark yellow, slightly raised spots, and sometimes the presence of white mycelium on fruit epicarp. Other fruit showed sunken, dry, tan lesions at the stylar-end, sometimes in conjunction with the other symptoms described above. Since Key lime is considered a new and emerging crop in Italy, especially in the Southern regions, it is crucial to identify

pathogens and limiting factors for this crop in order to properly manage the cultivation. The aim of this study was to investigate the etiology of the lesions observed in both phases of pre-harvest as well as post-harvest.

8.2 Materials and Methods

8.2.1 Isolation

The incidence of symptomatic fruit in field was determined on approximately 40 plants, randomly selected. Fifty symptomatic fruit samples were brought to the Plant Pathology laboratory of the Dipartimento di Agricoltura, Alimentazione e Ambiente, Sezione di Patologia vegetale, University of Catania for further investigations. Small sections (0.5×0.5 cm^2) of diseased albedo and flavedo tissues were surface-disinfected for 1 min in 1.5% sodium hypochlorite, rinsed in sterile water, placed on potato dextrose agar (PDA, Lickson) amended with 100 mg/liter of streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) to prevent bacterial growth, and then incubated at 25 ± 1 °C for three–four days. Representative single-spore isolates of fungal colonies were obtained from pure cultures grown on PDA at 25 ± 1 °C.

8.2.2 Morphological and Molecular Characterization

For the morphological characterization of the pathogen, the length and width of 30 arthroconidia from the seven-days-old colony of the isolate Geo1 grown on PDA were measured using a fluorescence microscope (Olympus-BX61) coupled to an Olympus DP70 digital camera; images and measurements were captured using the software analySIS Image Processing. Dimensions are reported as

the minimum and maximum in parentheses, and the average is reported with the standard deviation. Representative isolates were stored in the Plant Pathology collection of the Dipartimento di Agricoltura, Alimentazione e Ambiente, Sezione di Patologia vegetale, University of Catania. Genomic DNA of the selected isolates (Geo1, 2, 5, 8, 9 and 11) was extracted using the Gentra Puregene Yeast/Bact. Kit (Qiagen), Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), and also directly extracted by Macrogen Inc. (Seoul, South Korea). The internal transcriber spacer region (ITS) of the nuclear ribosomal RNA cluster was repeatedly amplified with different primer combinations, using ITS1f/ITS5 and ITS4 (Gardes and Bruns, 1993; White *et al*., 1990). PCR amplification conditions were set as follows: initial denaturation temperature of 94 °C for 30 s, followed by 35 cycles at the denaturation temperature of 94 °C for 30 s, annealing temperature of 50–52 °C for 1 min, extension at 68 °C for 1 min, and final extension at 68 °C for 5 min. PCR products were purified and sequenced in both directions by Macrogen Inc. (South Korea). The same region was also sequenced by Macrogen Inc. (South Korea) using primers ITS1/ITS4 (White *et al*., 1990). Sequences were read and edited using MEGAX: Molecular Evolutionary Genetics Analysis (Kumar *et al*., 2018). BLAST searches were performed against the NCBI nucleotide database (Altschul *et al*., 997).

8.2.3 Pathogenicity Test

In order to fulfil Koch’s postulates, a total of 18 fruit for each treatment condition were used in the pathogenicity tests. Two inoculation sites were used for each fruit at the stylar-end. Five fruit for each treatment were used as control. Treatment conditions consisted of: (a) over-ripe Key limes; (b) ripe lemons “Femminello

Siracusano 2 KR"; and (c) ripe (green) Key limes. Each treatment was incubated at 25 °C, and treatments a and b were also incubated at 4 °C. Fruit were surface-disinfected in 2% sodium hypochlorite solution for 10 min and rinsed twice in sterile deionized water. Once completely air-dried on a laboratory bench, two wounds were made with a needle (insulin syringe) at the stylar-end on the opposite sides respectively, and 20 µL of 10^6 arthroconidia/mL suspension of the isolate Geo1 were pipetted onto two inoculation sites. Controls consisted of wounded fruit inoculated with sterile water only. Replicates were kept in plastic containers to maintain a high humidity in a growth chamber with a 12 h photoperiod at 25 ± 1 °C and in the refrigerator at 4 °C. The disease incidence, indicated as the percentage (%) of fruit showing a rotten area in at least one inoculation site, was recorded 48 h and seven days after inoculation. Reisolations were conducted as described above from representative inoculated fruit and from controls in order to fulfill Koch's postulates.

8.3 Results

Field survey conducted in November of 2020 in three hectares of commercial orchard of Key lime (~1300 trees) cultivated under shade netting in Catania Province, Sicily (Italy) showed the presence of almost 15% symptomatic fruit. Limes still attached to the trees, as well as those already harvested, showed sunken and dry lesions at the stylar-end, often covering up to half of the fruit surface (Figure 27a–c). Symptoms of water-soaked spots at the stylar-end were also observed on attached and harvested fruits, often showing white mycelium on the rotten area (Figures 27d–f and 28a). Fruit showing SEB symptoms, once cut in half, showed the presence of translucid areas close to the stylar-end and the mammiform tip. From both kinds

of symptoms, a filamentous yeast-like fungus was consistently isolated, having a thin, flat, white-to-cream, lightly powdery mycelium, firstly identified as *Geotrichum*-like (Figure 28b). Arthroconidia were hyaline, sub-globose-to-cylindrical in shape, ranging from (5.4-) 7.2 ± 1.1 (-10.4) × (3-) 4 ± 0.6 (-5.2) µm (Figure 28c). Regarding the molecular characterization, chromatograms resulting from the sequencing of partial internal transcriber spacer region (ITS) revealed the presence of dual peaks, especially in forwards. Multiple attempts were made in isolates' reculturing, DNA re-extractions and sequencing, but the amplicons always showed dual peaks, which was the reason why the BLAST search was conducted using the reverse sequences only, which seemed to be clean for at least 230 bp. Edited reverse amplicons (232 bp) showed 100% identity with *Galactomyces citri-aurantii* (asexual morph *Geotrichum citri-aurantii*) (GenBank accession MH153586). The results from the pathogenicity test, summarized in Table 6, reveal that only over-ripe limes incubated at 25 °C developed symptoms of water-soaked spots at 48 h (22%) and seven days (77%) after inoculation, as well as ripe lemons "Femminello Siracusano 2 KR" after seven days (55%) (Figure 2d). Some fruit developed white mycelium on the epicarp. None of the inoculated fruit developed sunken/dry lesions, typical symptom of SEB, at the stylar-end. Therefore, this result induced us to attribute this symptom to the physiological disorder known as stylar-end breakdown (SEB), whereas the water-soaked spots were attributed to the sour rot. Controls did not show any symptom. Reisolations from symptomatic limes and lemons showed 100% *G. citri-aurantii* and no fungal colonies from controls.

Figure 27 Symptoms on Key lime in pre-harvest. a–c, Sunken and dry lesions on Key limes (SEB); d-e, symptoms of sour rot; f, rotten limes on the ground covered by abundant white *Geotrichum mycelium*.

Figure 28 Sour rot details. a, Key limes after harvesting showing symptoms at the stylar-end (SEB and sour rot); b, 10-days-old colony of *Geotrichum citri-aurantii* isolate Geo1 on PDA; c, arthroconidia of *G. citri-aurantii* isolate Geo1 (scale bar = 50 µm); d, ripe (left) and over-ripe (right) Key limes seven days after inoculation. Black dots represent the inoculation sites.

Table 6 Treatment conditions and Disease Incidence (D. I.) in pathogenicity test.

Treatment Conditions	D.I. 48 h	D.I. 7 Days
Over-ripe lime 25 °C	22%	77%
Ripe (green) lime 25 °C	0%	0%
Over-ripe lime 4 °C	0%	0%
Ripe lemon 25 °C	0%	55%
Ripe lemon 4 °C	0%	0%
Control	0%	0%

8.4 Discussion

The results of the present study highlight unusual alterations of Key lime in pre- harvest condition. Field surveys revealed two different symptoms at the stylar-end: water- soaked spots covered with a yeasty, sometimes wrinkled layer of white-colored mycelium, and sunken/dry lesions. The results of isolations showed a high incidence of a yeast- fungus, characterized as *G. citri-aurantii*. The identity of the six isolates characterized based on morphological aspects was complemented by means of the partial internal transcribed spacer sequences. Difficulties in the sequencing of the ITS region, and the constant presence of dual peaks in the resulting amplicons after many sequencing efforts, emerged from this study. Other authors reported the same issues with *Geotrichum* spp., especially for the ribosomial DNA, which seemed to be characterized by a high heterogeneity (Alper *et* al., 2011; De Hoog and Smith, 2004; Groenewalde *et al.*, 2012). These authors constantly noticed the presence of dual peaks, and most of the analyzed strains were processed multiple times in order to avoid the possibility of some methodological mistake. The results of previous researches demonstrated the presence of many different ITS1-5.8S-ITS2 variants within the same strains (Alper *et* al., 2011; De Hoog and Smith, 2004; Groenewalde *et al.*, 2012). The similarity of our difficult sequencing of the ribosomial DNA led us to hypothesize intragenomic rDNA variability within our isolates, as previously confirmed (Alper *et* al., 2011; De Hoog and Smith, 2004; Groenewalde *et al.*, 2012). Sour rot of *Citrus* fruit was described in California in 1917 and was attributed to *Oospora citri-aurantii* (Smith, 1917), nowadays named *G. citri-aurantii* (Timmer *et al.*, 1999) a variety of *G. candidum* (Mycobank current name *G. candidum* var. *citri- aurantii* MB# 123736). Sour rot usually leads to a complete disintegration of the fruit due to the degrading activity of the

extracellular enzymes of the fungus in the rind, segment walls and juice vesicles (Timmer *et al*., 1999). The physiological disorder known as stylar-end breakdown has been deeply investigated, being one of the main limiting factors in lime production and commercialization. It is mainly considered a post-harvest disorder, but it occasionally occurs in pre-harvest under certain conditions: particularly with high temperatures after rainy events (Timmer *et al*., 1999) Suffice it to say that the lime industry in the US has deemed SEB its number one problem (Davenport *et al*., 1976). For many years, researchers thought that it was a rind disorder determined by a chain reaction of cellular breakdown that rapidly spread to the albedo and flavedo after the fruit struck the ground (Davenport *et al*., 1976). Many studies investigated the causes of this disorder, leading to the conclusion that the affected area was determined by the juice vesicles' rupture and chlorophyll destruction in the rind (Timmer *et al*., 1999). Further investigations revealed that the cause of the characteristic symptom at the stylar-end was the rupture of the juice vesicles and the passing of the juice into the rind (Davenport *et al*., 1976). Although there is no evidence of any microorganism responsible for SEB, but only physiological processes, it is interesting to underline the environmental conditions responsible for its occurrence and the possible association with some microorganism. The results of our pathogenicity tests revealed that *G. citri-aurantii* was responsible for the water-soaked spots at the stylar-end. Devenport *et al*. (1976) studied three factors that could contribute to the incidence of SEB, which were bruising, fruit maturity (size) and field heat, and tried to elucidate the mechanisms involved in the vesicles' rupture. One of the mechanisms could pertain to the weakening of vesicles' membranes and cell walls, and the consequent inability to withstand the turgor pressure; and another mechanism could pertain to the resistance limit point of the turgor and/or the internal fruit pressure. The authors concluded that excessive turgor pressure and heat stress associated

with fruit maturity were the causes of the disorder; thereby, they strongly recommend that one avoid over-ripening on the tree (Davenport *et al*., 1976). It is interesting to note that all these factors that are related to SEB disorder are the same that predispose limes to sour rot. As demonstrated for lemon fruit, the physiological age, storage time and water status of the fruit are the main factors influencing susceptibility to sour rot (Baudoin and Eckert, 19). Although we cannot affirm any relation of causality between the presence of *G. citri-aurantii* in fruit affected by SEB, we can indeed confirm, as previously affirmed (Rose *et al*., 1943), that fruit showing SEB symptoms facilitate the development of the sour rot. Our field survey revealed that lime fruit showing sour rot in pre-harvest were significantly over-ripe. Pathogenicity tests, in fact, confirmed that the highest percentage of disease incidence resulted in over-ripe limes incubated at 25 °C, whereas over-ripe limes incubated at 4 °C and ripe (green) limes incubated at 25 °C did not show any symptoms of sour rot. These results indicate that environmental and physiological factors are crucial in the disease development. Harvesting practices therefore become an important step in order to control SEB disorder and sour rot. The orchard investigated in our study showed different agronomic and environmental conditions important in fruit diseases/alterations. Trees were grown under shade netting, and this, with respect to an open-air system, could provide suitable conditions for the pathogen in terms of humidity and canopy ventilation, along with the heavy clay/lime soil and the presence of infected fruit left in the orchard. As demonstrated in California, where environmental conditions for sour rot of peach and nectarine infrequently occur, a high soil pathogen population, the presence of fallen fruit remaining on the ground and episodes of high humidity led to sour rot in the field (Yaghmour *et al*., 2012). Our field observations revealed the presence of infected fruit that remained on the ground or attached to the trees. This condition, in addition to the presence of rain or insects, represents

a critical situation for the growers. The dispersal of inoculum vectored by insects is reported for this pathogen, mainly carried on the body surface of fruit flies and nitidulid beetles (Yaghmour *et al*., 2012), and therefore it is strongly recommended that one discard infected fruit from the orchard. *Geotrichum candidum* is widely reported to be found in the soil (Butler and Eckert, 1962; Hershenhorn *et al*., 1992), and this represents an important source of inoculum, especially in the case of soil contamination of the fruit packing line from the orchard to the packinghouse (Yaghmour *et al*., 2012). Investigating the *Geotrichum* spp. soil population is relevant in terms of its management. Studies conducted in California demonstrated a decline of the *G. candidum* population with an increasing soil depth (Yaghmour *et al*., 2012). Both sour rot and SEB are considered mainly post-harvest diseases. The results of our study highlight how environmental and agronomic conditions are very important in preventing important diseases and/or alterations.

8.5 Conclusions

This study underlines an unusual presence in pre-harvest of stylar-end breakdown and sour rot caused by *G. citri-aurantii* on Key lime, usually considered post-harvest diseases. Environmental conditions and agronomic practices could be crucial to prevent the occurrence of both alterations on this crop, which represents an important economic income for growers in Italy and Mediterranean countries. In addition, to our knowledge, this is the first report of *G. citri-aurantii* on Key lime in Europe.

8.6 References

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9. General conclusion

This Ph.D. thesis is the results of three years of investigation on fungi belonging to the *Botryosphaeriaceae* family in Sicily, Italy, with a specific focus on Mediterranean and Tropical crops. Over the last decade, the incidence of *Botryosphaeriaceae* disease has been steadily increasing worldwide, with numerous reports now present in the literature. This fungal families exhibit a polyphagous nature, enabling many species to adapt to diverse environmental conditions, that easily jumping from one host to another. Furthermore, *Botryosphaeriaceae* can be isolated from native hosts within an area and also infect introduced hosts in the same area. Climate change have significant implications in the diffusion and behavior of plant pathogens, and the extreme weather events create a stress condition advantageous for *Botryosphaeriaceae* infections. The global spread is further facilitated by the international movement of plants and derivates, without proper quarantine rules system. In open field, as well as in the urban areas and nurseries, the spread is favored by spore dispersion in a short distance by splash rain, wind, insects, and pruning operations done in a favorable condition for infections. In Italy, as well as in all the world, the relevant presence of *Botryosphaeriaceae* infections observed during these years and its high diffusion among different agricultural and ornamental crops, revealed the importance of this group of fungi that are becoming a limiting factor for plant production. Research conducted in several agricultural holdings, nurseries, and urban areas as part of this thesis, highlighted the disease symptoms observed on Mediterranean and tropical crops, with particular focus on avocado, Indian laurel-leaf fig and *Acacia* spp., and has contributed to elucidate the etiology of emerging disease affect tree crops in Sicily (Italy).

In the Experimental part 1 we investigate the most avocado productive area of Sicily. We studied the etiology of the symptoms, and we described six species based on morphology and multi-loci

phylogenetic analyses: *Botryosphaeria dothidea, Lasiodiplodia citricola*, *Macrophomina phaseolina, Neofusicoccum cryptoaustrale*, *Neofusicoccum luteum* and *Neofusicoccum parvum.* This study provides an update result on the association of *Botryosphaeriaceae* species on avocado and in particular, in accordance with the international literature, reporting *L. citricola*, *M. phaseolina* and *N. cryptoaustrale* causing canker and dieback on avocado trees worldwide and is the first report of all the recorded fungi causing branch disease on avocado in Italy.

As previously mentioned in the introduction section, we also studied disease symptoms on ornamental trees. In the Experimental part 2 we investigated disease symptoms in the street-lined tree, squares, gardens and parks, mainly in Catania and Siracusa province of Sicily, Italy. The survey highlighted the presence of canker and dieback symptoms on Indian leaf-laurel fig, particularly in trees severely and improperly pruned during the humid season. Morphological and molecular characterizations of the recovered fungal isolates confirm the presence of *Botryosphaeria dothidea*, *Neofusicoccum mediterraneum* and *Neofusicoccum parvum*. The results demonstrated the existence of a new disease on *Ficus microcarpa* in Europe.

In Experimental part 3 we investigated disease symptoms on *Acacia* species, in detail *A*. *dealbata* grafted on *A*. *retinodes,* both growth in a nursery located in Messina province (Sicily, Italy). Diseased plant exhibited necrotic sunken lesion at the stem level and graft union. Through our studies we confirmed the presence of the *Botryosphaeriaceae* species, *Lasiodiplodia citricola*. The cultivation process has an impact on disease incidence, in particular the irrigation with sprinklers increases the environmental conditions for spore dispersion, and the use of infected tools can easily promote infection. This study represents the first report of *L. citricola* causing shot blight and canker on *A*. *dealbata* and *A*. *retinodes* worldwide.

In the three Ph.D. years, extra research activities were conducted, and

we characterized other fungal taxa causing disease on innovative crops in Sicily. During the surveys conducted on avocado, we found disease symptoms on the stem of young plants, and at the crown and root of young potted plants. The isolations from symptomatic tissue yielded *Neopestalotiopsis* and *Rosellinia*-like colonies, respectively. Results of morphology and phylogeny reveled three species, *Neopestalotiopsis rosae* and *Neopestalotiopsis siciliana* sp. nov. from stem and branch symptoms, while *Rosellinia necatrix* from the crown and root symptoms. These studies represent a first report of *N. rosae* and the novel species *N. siciliana* affect avocado worldwide, and also the first report of *Rosellinia necatrix* causing white root rot in Italy. Meanwhile, other research activities were conducted on innovative crops, such as passion fruit and key lime, where disease symptoms were notified and recorded. Our characterization studies, based on morphology and phylogeny, reveal the presence of *Fusarium nirembergiae* and *Geotrichum citri-aurantii* species, respectively.

In conclusion, based on our results we described many *Botryosphaeriaceae* species in Italy. The knowledge acquired will may help to study the ecology of that fungi and also to develop a control strategy in order to limit the impact of them in agricultural system and urban landscapes. The research conducted in the present thesis shown that species belonging to the *Botryosphaeriaceae* represent key pathogens of the patho-system studied and can strongly affect the quality and quantity of the production as well as the integrity of natural ecosystems.

10. Annexes: Scientific Curriculum

10.1 Research and professional experience

- May 2023-on going: Working collaboration with Battaglio S.p.a. regarding surveys and economic reports of *Citrus*, tropical and pome farming in Sicily, Basilicata and Emilia-Romagna. Focus on strengths, weaknesses, threats, and opportunities.

- April-Nov 2022: Kearney Agricultural Research and Extension Center, University of California – Davis, USA. Research project: "Developing a qPCR system for quantification of shoot infection level of *Botryosphaeriaceae* fungi in avocado plants". Under the supervision of Dr. Themis Michailides and Dr. Yong Luo.

- 2020-2023: PhD course in Agricultural, Food and Environmental Science, University of Catania. Research project: "Diversity of *Botryosphaeriaceae* species on Mediterranean and Tropical plants". Under the supervision of Prof. Giancarlo Polizzi and Dr. Dalia Aiello.

10.2 Education and Professional qualifications

- May 2020: Qualification as Agronomist and Forestry Doctor. Board of Agronomists and Foresters of Catania (Italy).

- 2017-2019: MSc in Agricultural Sciences and Technologies LM69, specialty on Phytosanitary Technologies - University of Catania, Catania (Italy). Experimental thesis: "Characterization of

*Eutypa l*ata and *Cytospora pistaciae* causing dieback and gummy canker of pistachio in Italy".

- 2013-2017: BSc in Agricultural Sciences and Technologies L25 - University of Catania, Catania (Italy). Thesis: " Mineral nutrition of potted plant in the nursery".

- 2013: High School Graduation: IISS "Benedetto Radice", Bronte (Italy).

10.3 Scientific contributions

- **Fiorenza A.**, Gusella G., Vecchio L., Aiello D., Polizzi G. (2023). Diversity of *Botryosphaeriaceae* species associated with canker and dieback on Avocado in Italy. *Phytopathologia Mediterranea*, 62(1), 47-63.

- Costanzo M.B., Gusella G., **Fiorenza A.**, Leonardi G.R., Aiello D., Polizzi G. (2022). *Lasiodiplodia citricola*, a new causal agent of *Acacia* spp. dieback. *New Disease Reports*, 45(2), e12094.

- **Fiorenza A.**, Gusella G., Aiello D., Polizzi G., Voglmayr H. (2022). *Neopestalotiopsis siciliana* sp. nov. and *N. rosae* Causing Stem Lesion and Dieback on Avocado Plants in Italy. *Journal of Fungi*, 8(6), 562.

- **Fiorenza A.**, Aiello D., Costanzo M.B., Gusella G., Polizzi G. (2022). A New Disease for Europe of *Ficus microcarpa* Caused by *Botryosphaeriaceae* Species. *Plants*, 11(6), 727.

- **Fiorenza A**., Aiello D., Leonardi G.R., Continella A., Polizzi G. (2021). First Report of *Rosellinia necatrix* Causing White Root Rot on Avocado in Italy. *Plant Disease*, PDIS-02.

- Aiello D., **Fiorenza A**., Leonardi G.R., Vitale A., Polizzi G. (2021). *Fusarium nirenbergiae* (*Fusarium oxysporum* Species Complex) Causing the Wilting of Passion Fruit in Italy. *Plants*, 10, 2011.

- Gusella G., **Fiorenza A**., Aiello D., Polizzi G. (2021). Unusual Stylar-End Breakdown and Sour Rot on Key Lime (*Citrus aurantiifolia*) in Pre-Harvest Condition in Italy. *Plants*, 10, 989.

- Aiello D., **Fiorenza A**., Gusella G., Polizzi G. (2020). First report of *Calonectria tunisiana* causing crown and root rot on *Eucalyptus globulus*. *Journal of Plant Pathology*, 102, 1353-1353.

- Aiello D., Gusella G., **Fiorenza A**., Guarnaccia V., Polizzi G. (2020). Identification of *Neofusicoccum parvum* causing canker and twig blight on *Ficus carica* in Italy. *Phytopathologia Mediterranea*, 59(1), 213-218.

- Aiello D., Polizzi G., Gusella G., **Fiorenza A**., Guarnaccia V. (2019). Characterization of *Eutypa lata* and *Cytospora pistaciae* causing dieback and canker of pistachio in Italy. *Phytopathologia Mediterranea*, 58(3), 699-706.