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CHARACTERIZATION OF NEW FUNGAL DISEASES OF PISTACHIO IN SICILY

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“*The most telling and profound way of describing the evolution of the universe would undoubtedly be to trace the evolution of love*.”

Pierre Teilhard de Chardin

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Research highlights

- Pistachio production in Sicily represents an important economic income for the national production.
- Many diseases can infect pistachios around the world.
- Fungal diseases represent a serious risk for this crop, from the field to the storage.
- Many organs of the tree can be infected, from the canopy to the root system.
- The canopy can be severely attacked by *Septoria*-like pathogens and by *Botryosphaeriaceae* spp.
- Fungi belonging to the *Botryosphaeriaceae* fam. are considered the most destructive and dangerous pathogens for pistachio.
- New molecular approaches need to investigate the taxonomy of all these species.
- Investigation and characterization of the pathogen's population in the different production areas of the island are needed to clarify the phytopathological situation of this crop in the island and in the Mediterranean area.

Thesis outline

Chapter 1: It is an introductive chapter that focuses on pistachio fungal diseases described worldwide. This introduction is divided in 4 paragraphs, specifically: the major fungal diseases worldwide, the minor fungal diseases reports, the phytopathological situation of pistachio in Sicily and the literature references. This chapter wants to provide the state of art of fungal diseases before to start with the experimental part described in the following chapters.

Chapters 2-4: In chapter 2, 3, and 4, it is described the experimental part regarding pistachio diseases in Sicily. Specifically, the morphological and molecular characterization of the fungal isolates recovered from symptomatic pistachio orchards in Sicily. In chapter 2 I provide updates regarding the "leaf spot disease" caused by *Septoria pistaciarum*. In chapter 3 the etiology of the "Botryosphaeria Panicle and Shoot Blight" disease, found in the new plantings in Sicily, was elucidated. Chapter 4 shows results regarding canker and dieback symptoms caused by *Cytospora pistaciae* and *Eutypa lata*. These studies were published in *Fungal Biology* (Doi: 10.1016/j.funbio.2021.08.006), *Plant Disease* (Doi: 10.1094/PDIS-08-21-1672-RE), and *Phytopathologia Mediterranea* (Doi: 10.13128/Phyto-10880), respectively.

Chapter 5: From chapter 5 to the end of the thesis is presented my research activity developed during the PhD mostly on other crop/diseases. In this chapter I present two studies on new diseases reported for the first time: *Arthrinium xenocordella* causing fruit blight on pistachio and *Neofusicoccum parvum* causing leaf and twig blight on the ornamental species *Rhaphiolepis indica*. These diseases notes were published in *Plant Disease* (Doi: 10.1094/PDIS-02-18-0290-PDN) and *Journal of Plant Pathology* (Doi: 10.1007/s42161-019-00412-5).

Chapters 6-7-8: In these chapters it is presented my research activity on *Botryosphaeriaceae* diseases on other fruit crops. *Botryosphaeriaceae* diseases

involve several taxonomic entities, symptoms and organs of the trees. Due to the polyphagous nature of the species involved in these complex diseases, and the aggressiveness of some species, these are considered serious threats for many fruit trees around the world. During these years of my PhD many symptomatic samples infected by *Botryosphaeriaceae* have been brought to our laboratory and analyzed, revealing new phytopathological cases. Specifically, chapter 6 is the result of a research conducted on English walnut infected by *Botryosphaeriaceae* spp. (*Forest Pathology* Doi: 10.1111/ efp.12661). Chapter 7 is the result of studies conducted in California about the "Fig limb dieback" caused by *Neoscytalidium dimidiatum* (*Plant Disease* Doi: 10.1094/PDIS-06-20-1226-RE). Chapter 8 shows the results of an investigation conducted in a nursery on the ornamental species *Brachychiton* spp. severely attacked by *Neofusicoccum parvum* (*European Journal of Plant Pathology* Doi: 10.1007/s10658-021-02379-5).

Chapter 9: In chapter 9 is presented the research activity conducted on avocado plants showing crown and root rot. Morphological characterization and multi-locus phylogenies using five genomic loci identified *Cylindrocladiella peruviana* and *Pleiocarpon algeriense* involved in the disease. This study is the first to report of stem and crown rot on avocado caused by *Cylindrocladiella peruviana* and *Pleiocarpon algeriense* (*European Journal of Plant Pathology* Doi: 10.1007/s10658-020-02082-x).

1. Introduction

1.1 Outlook on major fungal diseases of pistachio

Pistacia vera L. is a xerophytic, deciduous and dioecious tree belonging to the family *Anacardiaceae*. Due to the edibility and commercially acceptable dimension of the seeds it is the only species in the genus *Pistacia* successfully grown in orchards (Al-Saghir, 2009).

Native from the arid zones of Central Asia, its cultivation extended to Mediterranean regions of Southern Europe, North Africa, Middle East, China, United States and Australia (Hormaza *et al.,* 1998).

Although pistachio is considered a crop able to grow in difficult environmental conditions like hot, dry, desert-like areas, several diseases have been reported on this crop over the years.

Verticillium wilt caused by *Verticillium dahliae* Kleb, root and crown rot by *Phytophthora* spp. and Armillaria root rot by *Armillaria mellea* (Vahl.) are known as soilborne diseases affecting pistachio production areas (Holtz and Teviotdale, 2016; Eskalen *et al.,* 2001).

Once the research developed resistant rootstocks to control these pathogens, new studies investigated other diseases, revealing that this crop was susceptible to other pathogens, sometimes highly destructive.

Botryosphaeria Panicle and Shoot Blight is considered the main disease of this crop. Fungi belonging to the *Botryosphaeriaceae* fam. are well known in many agricultural, forestry and ornamental hosts, being pathogens, saprophytes, and endophytes (Slippers and Wingfield, 2007). Traditionally the morphological characteristics of conidia and colonies were widely used for the identification. In recent years, the phylogenetic concept of species, which includes DNA sequence homology and phylogenetic analysis improved the

knowledge of many cryptic species, previously linked to a single morphological species (Taylor *et al.,* 2000; Zhang *et al.,* 2021). *Botryosphaeriaceae* show 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). *Botryosphaeria dothidea* was reported on pistachio in California in 1984 (Rice *et al*., 1985), and later was deeply investigated on its biology, ecology, and epidemiology (Michailides, 1991; Michailides and Morgan, 1992; 2004). Nowadays, following the DNA-based molecular tools we know that many species within the *Botryosphaeriaceae* family are involved in the disease and reported worldwide, including *B. dothidea*, *B. rhodina*, *Diplodia seriata*, *Dothiorella iberica* (from now on: *Dot*.), *Dot. sarmentorum*, *Dot. viticola*, *Lasiodiplodia americana*, *L. citricola*, *L. gilanensis*, *L. pseudotheobromae*, *L. theobromae*, *Macrophomina phaseolina*, *Neofusicoccum australe*, *N. hellenicum*, *N. mediterraneum*, *N. parvum*, and *N. vitifusiforme* (Armengol *et al.,* 2008; Chen *et al.,* 2014, 2015; Crous *et al.,* 2000; Cunnington *et al.,* 2007; Holevas *et al.,* 2000; Inderbitzin *et al.,* 2010; López-Moral *et al.,* 2020; Mohammadi *et al.,* 2015; Nouri *et al.,* 2019; Saavedra-Opazo, 2011; Sohrabi *et al.,* 2020; Swart and Botes, 1995; Wunderlich *et al.,* 2012).

These fungi can attack several organs of the tree during different physiological stages. Symptoms on bark and xylem are cankers and discolouration (Michailides and Morgan, 2004). Since all the other organs (shoots, inflorescence, leaves, rachis and fruit clusters) are subject to different symptoms, depending on the seasonal period, development stage, and other factors involved in the disease development, (Ntahimpera *et al.,* 2002; Moral *et al.,* 2017), this disease is considered one of the main destructive. All the symptoms observable during the different seasons are summarized in Table 1 and Figure 2A-B. Based on the weather, symptoms can first appear in different months. Symptoms will first appear earlier in wet years than

in dry years.

Perennial nature of the host, and generally, Mediterranean climates influence the disease cycle. In polyetic epidemics, because of the inoculum of one season in generating primary inoculum during the next season (Zadoks and Schein, 1979), *Botryosphaeriaceae* diseases may extend for several successive years (Moral *et al.,* 2019).

Overwintering inoculum (conidia within pycnidia, and rarely ascospores from other hosts) already present within buds, cankers, rachises, petioles, and fruit mummies, start the primary infection in springtime, infecting young shoots, female panicles, and male blossoms. From the primary source of inoculum and/or from the infected organs during the spring it is possible to initiate a secondary infection during summer. Infected panicles, leaves, bud scales provide other inoculum during the growing season (Summer) and the dormant period (Fall and Winter) to enable the pathogen to start new cycles in the next year (Michailides and Morgan, 2004). Dispersal of the pathogen propagules are mainly guaranteed by the rain, sprinkler water (splash-dispersed pathogen) (Michailides and Morgan, 1993) and insects (Michailides and Morgan, 2016). Details of the disease cycle are shown in Figure 1.

Although cankers and wood decline have been widely investigated through the years in relation to the *Botryosphaeriaceae* species, little is known regarding the other fungal species involved in canker diseases of pistachio. In California, Nouri *et al.* (2019) investigated symptoms such as central discoloration of wood and bark, necrosis of vascular tissues and dead phellogen, identifying eleven fungal species in eight different genera in seven different families. Excluding the *Botryosphaeriaceae* spp., the author identified *Colletotrichum karsti*, *Cytospora californica* (from now on: *Cy.*), *Cy. joaquinensis*, *Cy. parapistaciae*, *Cy. pistaciae*, *Diaporthe ambigua*, *Didymella glomerata*, *Phaeoacremonium canadense*, and *Schizophyllum commune*. Among these, all the species resulted pathogenic, showing different level of virulence, with *Neofusicoccum*

(*Botryosphaeriaceae*) and *Cytospora* spp. (*Valsaceae*) being the most widespread and virulent. Recently, also field surveys conducted in Spain and Iran revealed the presence of fungi such as *Diaporthe neotheicola*, *Eutypa lata, Eutypella citricola, E. vitis, Phaeoacremonium cinereum* (from now on: *Ph.*), *Ph. minimum*, *Ph. parasiticum*, *Ph. viticola*, associated with cankers, branch dieback and shoot blight (López-Moral *et al.,* 2020b; Mohammadi *et al.,* 2015; Sohrabi *et al.,* 2020). Moreover, in Iran, which is considered one of the main production countries, the species *Paecilomyces variotii*, was identified as the causal agent of pistachio dieback which symptoms consist of cluster, leaf, and bud wilting, bark and wood discoloration, canker, and dieback (Alizadeh *et al.,* 2000). Recent phylogenetic studies on this genus elucidated the taxonomy of the causal agent, nowadays identified as *P. formosus* (Heidarian *et al.,* 2018). Presence of many different fungal taxa involved in canker disease represent an important element in terms of epidemiology and disease management. Most of these species have been found from other fruit and nut trees; frequently cultivated close to each other, increasing the assumption of the cross infections among these crops. Canker diseases represent a serious risk for older pistachio orchards where trees are easily subjected to different stress. Infact, stressed trees are more susceptible to infection by canker-causing fungal pathogens (Colhoun, 1973; Desprez-Loustau *et al.,* 2006; Ma *et al.,* 2001). Canker diseases California and in the Mediterranean area have been of increasing concern in the last 10 years (Chen *et al.,* 2014; Nouri *et al.,* 2018; Urbez-Torres *et al.,* 2010, 2013).

Wilted shoots (shepherd's hook), shriveled and dried leaves attached on the twig (flagging) are the main symptoms related to *Botrytis cinerea*, causal agent of Botrytis Blossom and Shoot Blight. Wood symptoms are cankers resulted from expansion of the fungus into older wood that can cause the death of 2-year-old wood, whereas infections on leaflets at the mid rib and upper part of the blade induce characteristic V-shaped lesions; on the contrary those on leaf blades

away from the midrib are large distinct, circular lesions with wide chlorotic margins (Ferguson and Haviland, 2016).

Source of inoculum of the pathogen are blighted shoots, contaminated buds (flower or vegetative), male inflorescence (on the tree or dropped on the ground), sclerotia, weeds and neighbor crops (Michailides and Morgan, 1994). The disease occurs during cool and wet springs and in the case of high humidity the fungus can sporulate releasing a grey mass of conidiophores on the attacked tissue. All the authors who described this disease on pistachio refer the higher susceptibility of the male trees (blossom blight) (Bolkan *et al.,* 1984, Elena and Vlahoyiannis, 2003) probably due to the larger and more compact inflorescences that favor retention of free water for longer period following rainfall or dew (Michailides, 1991).This disease, when severe, lead to excessive killing of current-season shoots (reduction of next-season's fruiting shoots) and blight of male inflorescence and consequent pollination problems (Ferguson and Haviland, 2016) (Figure 2C).

Very similar to the "Botrytis Blight", although sporadic, Sclerotinia Blight (*Sclerotinia sclerotiorum*) was found in 1986 in the San Joaquin and Sacramento Valleys, California (Michailides and Morgan, 1994). Symptoms like "shepherd's hook" and "flagging" are very similar to those described for *Botrytis* infections, but the only sign that could help to distinguish both is the presence of the sporulation of *B. cinerea*.

Alternaria Late Blight is another important disease affecting pistachio. It was reported for the first time in Egypt in 1974 (Wasfy *et al.,* 1974), in California in 1985 (Michailides *et al.,* 1994), in Italy (Corazza and Avanzato, 1986), South Africa (Swart and Blodgett, 1998), Australia (Ash and Lanoiselet, 2001), and in Turkey (Can *et al.,* 2014, Ozkilinc *et al.,* 2017). Although the taxonomy of this group of species is complicated due to the morphological plasticity, nowadays four species have been found in pistachio: *Alternaria alternata*, *A. arborescens*, *A. infectoria*, and *A. tenuissima*, (Pryor and

Michailides, 2002).

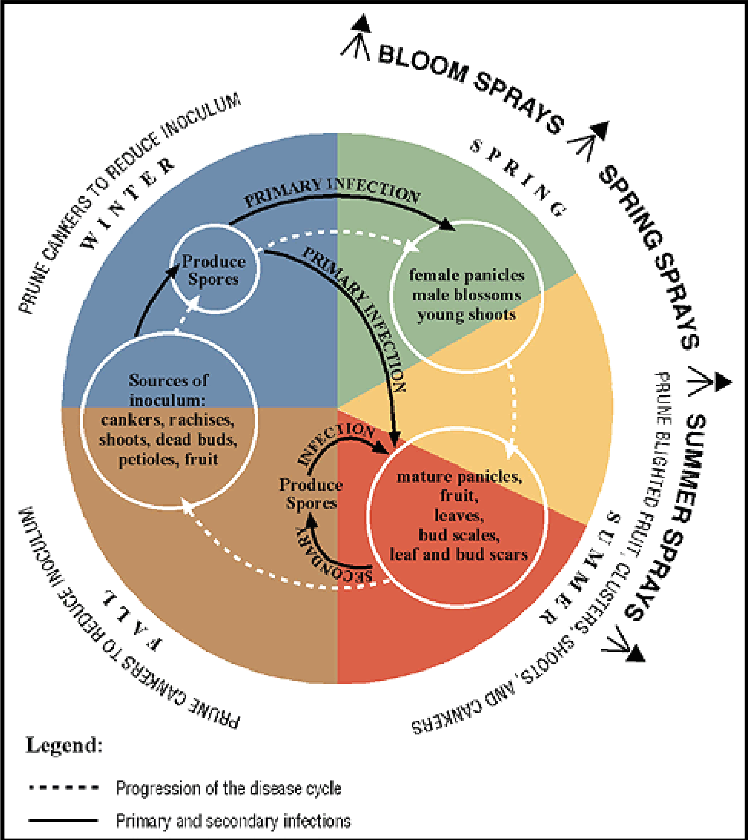
The name of the disease “Late Blight” was coined in California after the researchers observed an aggravation of the symptomatology in the late steps of pistachios maturation. Symptoms could both be present on the fruits epicarp as on the leaves as well. Infected fruits show small (1-2 mm) or large (~5 mm) black lesions surrounded with reddish-purple margins (Figure 2D), and early –split nuts can also be colonized by *Alternaria* resulting in staining shell (Figure 2E) (Michailides *et al.,* 1994). Whereas lesions on leaves are characterized by angular or circular black necrotic lesions (often with black sporulation in the center). Petioles, main veins, and leaf margins could be attacked too (Michailides *et al.,* 1994). Defoliation and quality reduction of the kernel are considered the worst damage attributed to the *Alternaria* attack.

Leaf spot diseases caused by *Septoria pistaciarum* and other *Septoria*-like pathogens are discussed in Chapter 2.

Table 1. Symptoms of Botryosphaeria Panicle and Shoot Blight (California Pistachio Commission, Botryosphaeria manual, January 1999).

Organs	Symptoms	Season
	lesion at shoot base	May-Jul.
Shoots	dying shoot	May-Aug.
	small black lesion	May-Jul.
Rachis	small black lesion (branch points)	Jul.-Aug.
	lesion at base of rachis	Jul.-Aug.
	cluster collapse	Aug.-Sep.
	gumming at the base of rachis	Aug.-Sep.
Leaves	mid rib infection of leaflets	Jun.-Aug.
	small black angular lesions	Jul.-Oct.
	dead leaflet infection leaflet base	Jul.-Aug.
	dead leaf infection base leaf stem	Jul.-Aug.
	large tan lesions on blade	Aug.
	defoliation	Aug.-Oct.
Fruit	young fruit clusters blighted	May- Jun.
	small black lesions (no red)	Jul.-Aug.
	associated with hemipteran wounds	Jul.-Aug.
	many tan dead nuts per cluster	Aug.
	silver-grey dead nuts per cluster	Aug.
	gum in older lesions	Aug.

Figure 1. Disease cycle of Botryosphaeria Panicle and Shoot Blight (Michailides and Morgan, 2004). Illustration: G. Conville



1.2 *Minor foliage and fruit diseases*

Regarding other minor diseases, Phomopsis Blight is reported in California and Greece. Symptoms are very similar to the Botrytis Blossom and Shoot Blight as well as Sclerotinia Blight.
It is considered a minor threat for pistachio production, but some experiments conducted inoculating current-season shoots (April) revealed the ability to induce leaves and petioles wilting within one week, and shoot blight in three weeks (Michailides, 2002).

The fungus *Beltrania pseudorhombica* (*Beltraniaceae*) was consistently isolated in 2016 from pistachio fruits in Arizona (U.S.), and in 2018 from infected leaves. Lichtemberg *et al*. (2019) described symptoms caused by *B. pseudorhombica* on pistachio (leaves and fruits) as dark brown spots surrounded by a pale brown halo. There are no other reports of this fungus attacking pistachio.

Pileolaria terebinthi and *P. pistaciae* are the causal agents of *Pistacia* spp. rust. These fungi are widely reported within the genus *Pistacia* in numerous world regions (Farr and Rossman, 2018). *P. terebinthi* (*Pileolariaceae*) is an autoecious macrocyclic fungus, characterized by typical lifestyle stages of these rusts.

Concerning recoveries of this fungus on *P. vera*, the spermogonial (0), aecial (I), uredial (II), telial (III), and basidial (IV) stages were reported around the world (Corazza and Avanzato, 1985; Chitzanidis, 1994; Dinc and Turan, 1975; Griggoriu, 1992; Petrak, 1956).

Teliospores are known as the overwintering structures on fallen leaves on the forest floor, germinating in late winter or early spring with the help of the early-season rain, and basidiospores as the primary infection initiators (Hamzehzargani and Banijashemi, 2002).

Specific symptoms are observable on leaves, petioles, pedicels and fruits showing round or irregular shaped, reddish-brown pustules, and generic symptoms like defoliation, blossom blight, branch deformity and die-back (Alaei *et al.,* 2012) (Figure 2F). Wind and

splashing water are considered the main vehicle for spores dispersion (Assaweh, 1969). Rust disease on pistachio was also described by Mirabile and Torta (2020) in Sicily, caused by *Tuberculina persicina*, anamorph of *Helicobasidium purpureum*. Fruits showed typical rusty lesions on the epicarp, releasing brown powdery mass of conidia (2G).

Very few and outdated data are available on powdery mildew. Reported as sporadic disease on pistachio it is caused by an unidentified powdery mildew species due to the lack of cleistothecia development (Michailides and Morgan 1994) (Figure 2H).

Old reports of the species *Phyllactinia guttata* (syn. *Phyllactinia suffulta*) come from Greece on *P. terebinthus* (Pantidou, 1973) and Sicily (Coucerio-Lopez *et al.,* 2013).

More recently Shin and Choi described *Phyllactinia pistaciae* sp. nov. on *P. vera* (Shin and Choi, 2003). Mycelium grows superficially as a white powdery mass, leaving brown scars after it dies. Symptoms can appear also on rachises, fruit stems, petioles, underside of leaf blades, and young shoots (Michailides and Morgan 1994).

Panicles and leaves can also be attacked by *Colletotrichum* spp. causal agents of the anthracnose hull rot. Symptoms are represented by black or pinkish lesions in the hulls. The disease is reported in California, Australia, and China (Hall *et al.,* 2014; Lichtemberg *et al.,* 2017; Yang *et al.,* 2011).

Regarding other occasional and minor pathogens reported on pistachio, in California *Phymatotrichopsis omnivora* was reported causing root rot, and *Pleurotus ostreatus* causing sapwood rot (French, 1989). *Aspergillus* spp., *Cladosporium* spp., *Epicoccum* spp., *Eurotium* spp., *Fusarium oxysporum*, *Fusarium* spp., *Penicllium* spp., *Phomopsis* and *Pestalotiopsis* sp., *Trichothecium* spp., *Uloclaudium* spp. and *Rhyzopus* spp. were reported related to various hull and kernel decays (Farr *et al.,* 2018; Ferguson and Haviland, 2016). Most of them are saprophytes fungi that live on dead organic matter in the orchard. Wind and blowing dust probably guarantee the spread of the

spores, with highest infection in seasons with significant rains in spring or just before and during harvest (Ferguson and Haviland, 2016).

Species belonging to *Aspergillus*, especially *A. niger*, are also associated to the sporadic disease named “Aspergillus Fruit Blight”. The disease occurs in very dry and hot years during the summer. Early symptoms are water-soaked discoloration of the hull (later soft and light beige); whereas at harvest fruits dry and the hull appear papery, densely wrinkled, and bright yellow (also the shell is bright yellow) (Figure 2I). Tissues under the epidermis are easily covered by black masses of spores, which leave, once washed off, brown or yellow stains on the shells. Presence of early-split or damaged nuts allow the spread of the disease. Sporulation of the fungus can be present in the nuts, leaves, shoots, and orchard floor under favorable conditions. (Ferguson and Haviland, 2016).

Moreover, in Texas (US), *Phomopsis lentisci* (*Phyllosticta lentisci*) (Anonymous, 1960), and in Maryland (US) *Bartalinia pistacina* (*Hyalotia pistacina)* (Maas, 1971) were reported associated to leaf spot, and *Corticium koleroga* (*Pellicularia koleroga*) to thread blight (Anonymous, 1960).

The ascomycete yeast *Nematospora* (*Eremothecium) coryli* and *Aureobasidium pullulans* are responsible for the Stygmatomycosis. Nuts infected by stigmatomycosis have a wet, smelly, rancid kernel (Ershad and Barkhordary, 1976; Ferguson and Haviland, 2016) (Figure 2L). Surveys conducted in California revealed three major symptoms of stigmatomycosis: 1) small, dark green, not fully developed kernels; 2) normal size kernel but wet, smelly, and rancid; 3) abnormal kernel white or light yellow and jelly-like (Michailides *et al.,* 2004).

Figure 2. Fruit symptoms. A-B. Botryosphaeria Panicle and Shoot Blight; C. Botrytis Blossom and Shoot Blight; D-E. Alternaria Late Blight; F. Rust (*Pileolaria terebinthi*); G. Rust (*Tuberculina persicina*); H. Powdery mildew; I. Aspergillus Fruit Blight; L. Stigmatomycosis.

1.3 Pistachio cultivation and diseases in Sicily

In Italy, pistachio production is centered in the southern regions, with Sicily considered as the first Italian producer. Specifically, the Catania Province is the main producer (3,583.6 t.), followed by Agrigento (2,36.2 t.), Caltanissetta (2,02.4 t.), Palermo (37 t.), and Messina (5.5 t.) provinces (http://dati.istat.it).

Currently, the commune of Bronte in Catania Province represents the most important area of Sicily for pistachio production, and this is an important economic resource for the territory (Barone and Marra, 2004). In this area, pistachio is grafted on spontaneous terebinth (*P. terebinthus*) plants which are grown on volcanic soils, hence the name of “natural pistachio plantings” (Barone *et al*., 1985) (Figure 3). Otherwise, mainly in Agrigento and Caltanissetta provinces, there are “new” orchards, characterized by a rational design, irrigation, fertilization, and mechanical harvest (Marino and Marra, 2019) (Figure 3).

Sicilian pistachio cultivars are: ‘Bianca’ (‘Napoletana’), ‘Femminella’, ‘Natalora’, ‘Agostana’. Other minor Sicilian cultivars are ‘Silvana’, ‘Cerasola’, ‘Cappuccia’, ‘Insolia’, ‘Ghiandalora’, ‘Gialla’, ‘Tardiva’ and ‘Pignatone’. Most of them are no longer grown nor available in nurseries (Marino and Marra, 2019). In general, Sicilian varieties are characterized by a unique organoleptic profile and high content of chlorophyll of the cotyledons (Marino and Marra, 2019). In Sicily, pistachio fruits of the traditional area of Bronte are protected by the label of PDO (protected designation of origin) “Green Pistachio of Bronte”, and pistachios from the new plantings of Agrigento and Caltanissetta provinces have been also recently protected by the PDO label of “Pistacchio di Raffadali”.

Regarding the phytopathological situation, few and outdated studies have been conducted to investigate pistachio diseases occurring in Italy. Passalacqua and Ciccarone in 1937 reported pistachio dieback in Agrigento area and they concluded that the

dieback was a result of no fertilization and especially no pruning practices followed by the growers (Montemartini, 1940). This dieback might refer also to studies that attributed some branch dieback in Campania region to the new species of *Botryodiplodia* (*B. pistaciae*) (Cristinzio, 1938). The *Botryodiplodia pistaciae* infections were named by Schilirò and Privitera (1988) as "pustule dei giovani rametti" that means "twigs with pustules", since this fungus forms longitudinal gray notches, and the necrotic bark is filled with little pustules. On the edges of the lesions the healthy tissues produce the cicatricial ones, and picnidia start to develop on the infected parts of the twigs. Relevant is the report in Sicily of the fungus *Cytospora terebinthi*, the causal agent of cankers and gummosis of young branches and trunk. Symptoms included oily spots soaked with gum on the cuticle of twigs during the spring, leading to serious branch cracking and formation of cankers (Corazza *et al*., 1990; Furnitto, 1984).

Corazza *et al*. (1986) reported the presence of *Botryosphaeria ribis* in Central Italy. In Southern Italy in 1995, some other types of symptoms were observed on leaves, cluster rachises, and fruits of pistachio trees. *Glomerella cingulata* (anamorph of *Colletotrichum gloeosporioides* was consistently isolated from naturally infected tissues, and further the artificial inoculations confirmed the similarity between symptoms observed in infected pistachio fruit in the field and artificially inoculated ones (Frisullo *et al*., 1996). The presence of the causal agent of pistachio anthracnose in Southern Italy could be an emergent threat for the recent, new Sicilian plantings.

Concerning canopy diseases of pistachio in Sicily, it is important to point out a report on the serious pathogen *Pileolaria terebinthi* the causal agent of pistachio rust (Berlese, 1897; Corazza and Avanzato, 1985; Corazza *et al*., 1990; Gualaccini, 1950). This pathogen alone could curb the Sicilian Pistachio industry from the nursery to the field (Rieuf, 1952).

At the same way, the pathogen *Alternaria alternata*, responsible

for necrotic lesions on the leaves that could lead to complete canopy desiccation and associated defoliation, has been reported in Italy on the "Napoletana" cultivar (Corazza and Avanzato, 1986) and has been signaled in Sicily in some orchard in Bronte territory (Schilirò and Privitera, 1988), showing no relevant consequences (Corazza *et al*., 1990).

Other diseases of minor importance in the Bronte area include *Phyllactinia suffulta* agent of powdery meldew and *Melampsora pistaciae* (Coucerio Lopez *et al*., 2013). *Phyllactinia suffulta* does not seem to be a dangerous threat under Sicilian climate (Schilirò and Privitera, 1988).

The fungus *Septogloeum pistaciae* is described as a pathogen able to attack the leaves and rarely the twigs of pistachio. It produces sub-epidermal acervuli that have no color as they ooze off the acervuli. (Schilirò and Privitera, 1988).

In regard to root diseases, we do not have significant reports except sporadic ones referring to the fungus *Armillaria mellea* (Corazza *et al*., 1990).

Old reports of diseases in Sicily (or in Italy) are summarized in Table 2.

Since pistachio cultivation is increasing in Italy, the Dipartimento di Agricoltura, Alimentazione e Ambiente, University of Catania, in recent years, started to deeply investigate fungal diseases affecting this crop. In 2018 a new canker pathogen has been described in the traditional area of Bronte, firstly described as *Liberomyces pistaciae*, and recently taxonomically reordered as *Leptosillia pistaciae* (Vitale *et al*., 2018; Voglmayr *et al*., 2019;). Other canker pathogens have been found involved in canker diseases, such as *Cytospora pistaciae*, and *Eutypa lata* (Aiello *et al*., 2019).

Regarding fruit diseases, recent reports in Italy concern *Arthrinium xenocordella* causing fruit blight, and *Tuberculina persicina* causing rust (Aiello *et al*., 2018, Mirabile and Torta, 2020).

Table 2 - Reports of pistachio diseases in Italy.

Pathogen	Symptoms	Reference
Alternaria alternata	Leaf lesions	Corazza and Avanzato, 1986
Armillaria mellea	White root rot	Corazza *et al*., 1990
Botryodiplodia pistaciae	Branch and twig dieback	Cristinzio, 1938 Schilirò and Privitera, 1988
Botryosphaeria ribis	Branch dieback	Corazza *et al.,* 1986
Cytospora terebinthi	Branch dieback	Corazza *et al.,* 1990; Furnitto, 1984
Glomerella cingulata	Anthracnose	Frisullo *et al*., 1996
Phyllactinia suffulta *Melampsora pistaciae*	Powdery mildew Powdery mildew	Coucerio Lopez *et al*., 2013
Pileolaria terebinthi	Rust	Berlese, 1897; Gualaccini, 1950; Corazza and Avanzato, 1985; Corazza *et al*., 1990
Septogloeum pistaciae	Leaf infection	Schilirò and Privitera, 1988
Septoria pistaciarum	Leaf spot	Caracciolo, 1934

Figure 2. – New orchards in Agrigento and Caltanissetta provinces.

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2. Experimental part: Update of pistachio leaf spot caused by *Septoria pistaciarum* in light of new taxonomic advances in Italy

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2.1 Introduction

Pistachio (*Pistacia vera* L.) is cultivated in the southern regions of Italy, of which Sicily is the main production area. The provinces of Catania, Agrigento and Caltanissetta are the largest pistachio producing areas in Italy. Currently, the commune of Bronte in Catania province is considered the most important area of Sicily for pistachio production, representing an important economic resource for the territory (Barone and Marra, 2004). In this area, pistachio is grafted on spontaneous terebinth pistachio (*Pistacia terebinthus*) plants which are grown on volcanic soils, hence the name of "natural pistachio plantings" (Barone *et al.,* 1985). However, mainly in Agrigento and Caltanissetta Provinces, there are "new" orchards, characterized by a rational design, irrigation, fertilization, and mechanical harvest (Marino and Marra, 2019). Few and outdated studies have been

conducted to investigate pistachio diseases occurring in Italy until the last two years. The most recent studies on pistachio diseases in Italy focused on canker pathogens, including *Cytospora pistaciae*, *Eutypa lata* and *Liberomyces pistaciae*, recently taxonomically reordered as *Leptosillia pistaciae* (Aiello *et al.,* 2019; Vitale *et al.,* 2018; Voglmayr *et al.,* 2019). Other recent reports in Italy described fruit pathogens, such as *Arthrinium xenocordella* and *Tuberculina persicina* (Aiello *et al.,* 2018; Mirabile and Torta, 2020).

Septoria leaf and fruit spot is one among the most important worldwide diseases on many cultivated and wild plants. *Septoria* s.l. represents a polyphyletic group of genera including many plant pathogens (Bakhshi *et al.,* 2019; Quaedvlieg *et al.,* 2013; Verkley *et al.,* 2013). Although the host-association has not to be considered a strict demarcating species factor, three different *Septoria* spp. have traditionally been associated with pistachio, including *Septoria pistaciae*, *Septoria pistaciarum* and *S. pistacina*. The oldest report of this disease date back to 1842 in France when Desmazieres (1842) described *S. pistaciae* causing leaf spot on *P. vera*. Allescher (1901) introduced *S. pistacina*, and some years later, Caracciolo (1934) reported a third species named *S. pistaciarum* in Sicily. In 1956, two sexual morphs, namely *Mycosphaerella pistacina* (for *S. pistacina*) and *Mycosphaerella pistaciarum* (for *S. pistaciarum*) have been reported by Chitzanidis (1956) in Greece. Several countries reported the presence of these species associated with pistachio, in particular, *S. pistaciarum* in Arizona, New Mexico (US), Greece, India, Italy, Spain, and in East-Mediterranean and Southeast Anatolian regions (Ahmad *et al.,* 2011; Caracciolo, 1934; Eskalen *et al.,* 2001; French *et al.,* 2009; Lopez-Moral *et al.,* 2021; Sarejanni, 1935; Young and Michailides, 1989), *S. pistaciae* in the United States (California), Italy, Greece, Ukraine and Egypt (Dudka *et al.,* 2004; Haggag *et al.,* 2006; Michailides, 1991; Montemartini, 1931; Pantidou 1973; Pupillo and Di Caro, 1952); and *S. pistacina* in Greece, Syria and Iran (Aghajani *et al.,* 2009; Chitzanidis, 1956; Spaulding, 1961). Recently, Crous et

al. (2013) elucidated the taxonomy of *Septoria*-like pathogens associated with pistachio, revealing three genera associated with this host, specifically *Cylindroseptoria*, *Pseudocercospora* and *Septoria s. str*. Until now, no molecular data from Italy confirmed the identity of Septoria spp. associated with *P. vera*. Due to the lack of a conspicuous number of gene sequences in published literature, and the outdated reports based on morphological characters only, the aim of this study was (a) to molecularly characterize representative isolates of *Septoria* spp. associated with pistachio leaf spot in Sicily, using a multi-locus approach in order to confirm the identity of the causal agent, and (b) to test the pathogenicity and provide updated information for further investigations.

2.2 Materials and methods

2.2.1 Field surveys and morphological characterization

During June of 2019, pistachio trees "Bianca" from three farms of Bronte area (Sicily, Italy), showing leaf spot symptoms were surveyed. Leaves showing red-brown spots with black margins were brought to the laboratory for isolation and further investigations. Field observations, and sampling of leaf material, were conducted in springtime (May), summertime (June) and also in autumn (November) in order to observe possible reproductive structures on leaf litter material. Observations were made using an Olympus SZX- ILLB2-200 stereoscope (Olympus, Tokyo, Japan). From the symptomatic samples, small pieces of 0.2-0.3 cm^2 were surface sterilized for 1 min in 1.5% sodium hypochlorite solution, rinsed with sterile water, air dried in a laminar hood and placed on Potato Dextrose Agar (PDA, Lickson, Vicari, Italy) amended with 100 mg/L of streptomycin sulfate (SigmaeAldrich, St. Louis, MO, USA). All the Petri plates were incubated at 25 ◦C for 7e10 days. The slowly-growing, septoria-

like colonies were transferred to PDA plates and single-hyphae isolates were obtained from pure cultures. Due to the very slow growth of the isolates, in order to obtain larger colonies in plates for further analysis, mycelial plugs were collected into 2.0 mL Microcentrifuge tubes with sterile deionized water and vortexed at high speed for 3e5 min. Few drops of the turbid suspension were streaked on PDA. Representative single-hyphae isolates from three different surveyed farms were stored in the fungal collection of the Dipartimento di Agricoltura, Alimentazione e Ambiente, Sezione di Patologia Vegetale, University of Catania. Six representative isolates namely SD6, SE, S1, S13, S14, and S15 were selected and used for further investigations. Isolates S13, S1 and S15 were also registered in the Westerdijk Fungal Biodiversity Institute (CBS culture collection), Utrecht, The Netherlands as follows: CBS 146141, CBS 146142, and CBS 146143, respectively.

A total of five artificial media were evaluated for the optimal mycelial growth, including: corn meal agar (CMA, Oxoid, UK), malt extract agar (MEA, Lickson, Vicari, Italy), oatmeal agar (OA, Difco, Detroit, MI, USA), PDA, and synthetic nutrient agar (per Liter of SNA, 1g of KH_2PO_4; 1g of KNO_3; 0.5g $MgSO_4 * 7H_2O$; 0.5g of KCl; 0.2g of Glucose; 0.2g of Saccharose; 20g of Bacto Agar) following recipe label instructions. A 5-mm plug of a 7-days old colony of isolate CBS 146142 (S1) was removed and transferred to the center of a 90-mm Petri plate, and the plates were incubated at 25 ◦C for 14 days. Four Petri plates were used for each tested media. The experiment was repeated once. Two perpendicular diameters were recorded after 14 days of incubation, and data were transformed to radial growth rate. Data were analyzed with ANOVA and mean differences were compared with Fisher's least significance difference test (LSD) at P 0.05 using Statistix 10 (Analytical Software, 2013). Additionally, some isolates were cultivated on water agar (WA) containing sterile stinging nettle (*Urtica dioica*) stems but were not considered as a treatment in this assay.

In order to study conidial morphology, a total of 50 conidia were collected from 7-days-old colonies of representative isolates grown on PDA. Length and width were measured using an Olympus-BX61 fluorescence microscope (Olympus, Tokyo, Japan) coupled to an Olympus DP70 digital camera; images and measurements were captured using the software analySIS 3.2 (Soft Imaging System GmbH, Münster, Germany) and DP Controller 1.1.1.89 (Olympus Optical Co., LTD).

2.2.2 *DNA extraction and amplification*

Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation, WI, USA) and Gentra Puregene Yeast/Bact. Kit (Qiagen). The internal transcriber spacer region (ITS) of the nuclear ribosomal RNA operon was amplified with primers ITS5 and ITS4 (White *et al.,* 1990), the primers EF1-728F (Carbone and Kohn, 1999) and EF-2 (O'Donnell *et al.,* 1998) were used to amplify part of the translation elongation factor 1-alpha gene (*tef1*), and primers set T1 (O'Donnell and Cigelnik, 1997) and β-Sandy-R (Stukenbrock *et al.,* 2012) were used for the partial beta-tubulin (*tub2*). PCR amplification conditions for ITS were set as follows: initial denaturation temperature of 94 ◦C for 5 min, followed by 35 cycles at the denaturation temperature of 94 ◦C for 30 s, annealing temperature of 48 ◦C for 50 s, extension at 72 ◦C for 2 min, and final extension at 72 ◦C for 7 min. PCR conditions for tef1 and tub2, were: 96 ◦C for 2 min, 40 cycles at 96 ◦C for 45 s (denaturation), 52 ◦C for 30 s (annealing), 72 ◦C for 90 s (extension) and final extension at 72 ◦C for 2 min. The PCR products were purified and sequenced in both directions by Macrogen Inc. (Seoul, South Korea).

2.2.3 *Phylogenetic analysis*

The DNA sequences generated were analyzed and computed

using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar *et al.,* 2018). Sequences were submitted to GenBank. The sequences obtained in this study were preliminarily blasted against the NCBIs GenBank nucleotide database to determine the closest relatives. Multiple sequences alignment was conducted based on the available recent literature (Crous *et al.,* 2013; Quaedvlieg *et al.,* 2013; Verkley *et al.,* 2013) using MEGA X. A total of 34 taxa were considered in the analysis, and *Dothistroma pini* isolates CBS 121005 and CBS 116485 served as the out- group (Table 1). Phylogenetic analyses were based on Maximum parsimony (MP) and Maximum Likelihood (ML). Sequences of ITS, *tef1* and *tub2* were concatenated using MEGA X, and manual adjustments of alignments were made when necessary. A partition homogeneity test with heuristic search and 1000 homogeneity replicates was performed using PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0a (Swofford, 2003) to test the discrepancy among the three genes dataset. The analysis of the combined dataset 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), rescaled consistence index (RC), and Homoplasy index (HI) were calculated. A total of 1000 bootstrap replicates were performed to test the robustness of the tree topology. For the ML analysis, MrModeltest v. 2.4 (Nylander, 2004) was used to identify the best-fit model of nucle- otide evolution for each gene according to the Akaike Information Criterion (AIC). The ML analysis of the combined genes was per- formed in GARLI v.0.951 (Zwickl, 2006), and clade support was assessed by 1000 bootstrap replicates.

Table 1. Information and GenBank accession numbers of the isolates used in the phylogenetic analysis. Isolates in bold are of the present study.

Species	Isolate no.	Host	Location	Collector	GenBank accession no.		
					ITS	*tef1*	*tub2*
Dothistroma pini	CBS 116485	*Pinus nigra*	USA	G. Adams	JX901739	JX901625	JX902196
D. pini	CBS 121005	*Pinus pallasiana*	Russia	T. S. Bulgakov	KF251155	KF253115	KF252653
Septoria astragali	CBS 109117	*Astralagus glycyphyllos*	Austria	G.J.M. Verkley	KF251349	KF253296	KF252821
S. astragali	CBS 123878	*Astralagus glycyphyllos*	Czech Rep.	G.J.M. Verkley	KF251350	KF253297	KF252822
S. hippocastani	CPC 23103	*Aesculus* sp.	Netherlands	S.I.R. Videira	KF251563	KF253510	KF253031
S. hippocastani	CBS 411.61	*Aesculus hippocastanum*	Germany	W. Gerlach	KF251435	KF253383	KF252907
S. justiciae	CBS 128610	*Justicia procumbens*	South Korea	H.D. Shin	KF251436	KF253384	KF252908
S. justiciae	CBS 128625	*Justicia procumbens*	South Korea	H.D. Shin	KF251437	KF253385	KF252909
S. lamiicola	CBS 109112	*Lamium album*	Austria	G.J.M. Verkley	KF251445	KF253393	KF252917
S. lamiicola	CBS 109113	*Lamium album*	Austria	G.J.M. Verkley	KF251446	KF253394	KF252918
S. pistaciae	CBS 420.51	*Pistacia vera*	Italy	G. Goidánich	KF251520	KF253469	KF252989
S. pistaciarum	CPC 23116; 5DMR032	*Pistacia vera*	Turkey	K. Sarpkaya	KF442651	KF442635	KF442737
S. pistaciarum	CPC 23114; 003c	*Pistacia vera*	Turkey	K. Sarpkaya	KF442652	KF442641	KF442738
S. pistaciarum	CPC 23115; 002B	*Pistacia terebinthus*	Turkey	K. Sarpkaya	KF442653	KF442642	KF442739
S. pistaciarum	CBS 135838; 45sln034	*Pistacia vera*	Turkey	K. Sarpkaya	KF442654	KF442643	KF442740
S. pistaciarum	CBS 135839; 001A	*Pistacia vera*	Turkey	K. Sarpkaya	KF442655	KF442644	KF442741
S. pistaciarum	**CBS 146141; S13**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ268214**	**MZ285907**	**MZ285913**
S. pistaciarum	**CBS 146142; S1**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ268215**	**MZ285908**	**MZ285914**
S. pistaciarum	**CBS 146143; S15**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ268216**	**MZ285909**	**MZ285915**
S. pistaciarum	**SD6**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ268217**	**MZ285910**	**MZ285916**
S. pistaciarum	**SE**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ268218**	**MZ285911**	**MZ285917**
S. pistaciarum	**S14**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ268219**	**MZ285912**	**MZ285918**
S. protearum	CPC 19691	*Zanthedeschia aethiopica*	South Africa	P.W. Crous	KF251525	KF253474	KF252994
S. protearum	CBS 113114	*Geum* sp.	New Zealand	G.J.M. Verkley	KF251510	KF253459	KF252980
S. protearum	CBS 119942	*Asplenium ruta-muraria*	Germany	G.J.M. Verkley	KF251512	KF253461	KF252982
S. protearum	CBS 135477; CPC 19675	*Zanthedeschia aethiopica*	South Africa	P.W. Crous	KF251524	KF253473	KF252993

S. protearum	CBS 164.78	*Nephrolepis* sp.	New Zealand	H.J. Boesewinkel	KF251513	KF253462	KF252983
S. protearum	CBS 179.77	*Myosotis* sp.	New Zealand	H.J. Boesewinkel	KF251515	KF253464	KF252985
S. protearum	CBS 364.97	*Skimmia* sp.	Netherlands	J. de Gruyter	KF251517	KF253466	KF252986
S. protearum	CBS 390.59	*Ligustrum vulgare*	Italy	M. Ribaldi	KF251518	KF253467	KF252987
S. rumicum	CBS 503.76	*Rumex acetosa*	France	H.*A. van* der Aa	KF251529	KF253478	KF252998
S. stellariae	CBS 102376	*Stellaria media*	Netherlands	G.J.M. Verkley	KF251574	KF253521	KF253042
S. stellariae	CBS 102378	*Stellaria media*	Netherlands	G.J.M. Verkley	KF251575	KF253522	KF253043
S. stellariae	CBS 102410	*Stellaria media*	Netherlands	G.J.M. Verkley	KF251576	KF253523	KF253044

Table 2. Measurements of conidia of three *Septoria pistaciarum* isolates from pistachio in Italy.

Isolate	Conidia
	Length × Width (μm)[a]
S13 CBS 146141	(23.9-) 36.9 ± 4.5 (—50.2) × (2.0-) 2.7 ± 0.5 (—3.8)
S1 CBS 146142	(21.8-) 45.2 ± 8.5 (—64.4) × (1.7-) 2.9 ± 0.5 (—4.2)
S15 CBS 146143	(32.5-) 42.4 ± 5.2 (—59.9) × (1.8-) 2.8 ± 0.6 (—4.2)

[a] L × W =length ×width, (minimum -) average ± standard deviation (-maximum).

2.2.4 *Pathogenicity test*

Pathogenicity test was conducted in an experimental field of the University of Catania (unsprayed orchard). A total of six trees "Bianca" grafted on *P. terebinthus* were randomly selected and one shoot on each selected tree having 25-35 leaves was inoculated by spraying an aqueous conidial suspension (8×10^5 spores/ml) on both sides of the leaves. Three shoots on three different trees (35-68 leaves) were sprayed with sterile deionized water and served as negative controls. Inoculated shoots were covered with transparent plastic bags for 48 h. Inoculum was prepared culturing the representative isolate CBS 146142 (S1) on PDA and OA for 5-7 days at 26 ± 1 ◦C. Disease incidence was evaluated as the per- centage (%) of leaves showing characteristic spots 9 and 23 days after the inoculation. In the second evaluation, 30 random lesions were measured (length and width) and observed with the stereoscope to detect the presence of any pycnidia.

2.3 Results

2.3.1 *Field surveys and morphological characterization*

Symptomatic samples collected in the fields showed irregular red lesions with black margins on both sides of the leaves, usually confined by leaf veins, and increasing slightly in size with time. Sometimes, symptomatic material showed the presence of cirri exuding from the lesions (Fig. 1a-e). Stereoscopic observations conducted on leaves collected during springtime and summertime, revealed the presence only of solitary (~2.00 mm) or aggregated pycnidia bearing pycnidiospores (conidia). Examination of leaves collected in November showed the presence of fruiting bodies, similar to pycnidia or spermogonia. In both cases no conidia and/or spermatia have been found inside the reproductive structures, a reason why it

was not possible to ascertain with confidence the identity of each of the reproductive structures. Results of isolations from symptomatic leaves showed the constant presence of a septoria-like fungus, characterized by slow growing, gray/black, immersed colonies (Fig. 1f). This fungus was able to grow on each medium, showing different pigmentation, specifically red to salmon on OA (Fig. 2a), faint white on SNA and CMA (Fig. 2b and c), white on WA containing sterile stinging nettle (Fig. 2d), and gray/black on MEA and PDA, sometimes with red pigmentation, depending on the isolate (Fig. 2e-i). Presence of conidia was observed on each tested medium, except on CMA and SNA where pycnidia formation was not observed. Differences between each medium are shown in Fig. 3. Yellow mucilaginous matrix exuded from black conidiomata, often covered by white mycelium, was observed after 7 days from cultures grown on PDA (Fig. 4a). Mucilaginous material was also observed from colonies grown on OA and MEA after 14 days of incubation at 25 ◦C (Fig. 4b and c). Conidiophores (= conidiogenous cells) ~ 10 × 3 µm. Conidia were hyaline, curved to falcate, showing 1 to 5 septa (Fig. 4d and e). Measurements results, summarized in Table 2, were compared to the taxonomic key provided by Crous et al. (2013).

Figure 1. Septoria leaf spot of pistachio caused by *Septoria pistaciarum*. a. Symptoms observed in the field (June); b. Details of leaf spot; c. Crystalline cirrus exuded from a lesion; d. Symptoms observed at the end of July (coalesced lesions leading to necrotic patches); e. Symptomatic senescent fallen leaf (November); f. Results of isolations showing constant presence of the same Septoria-like colony

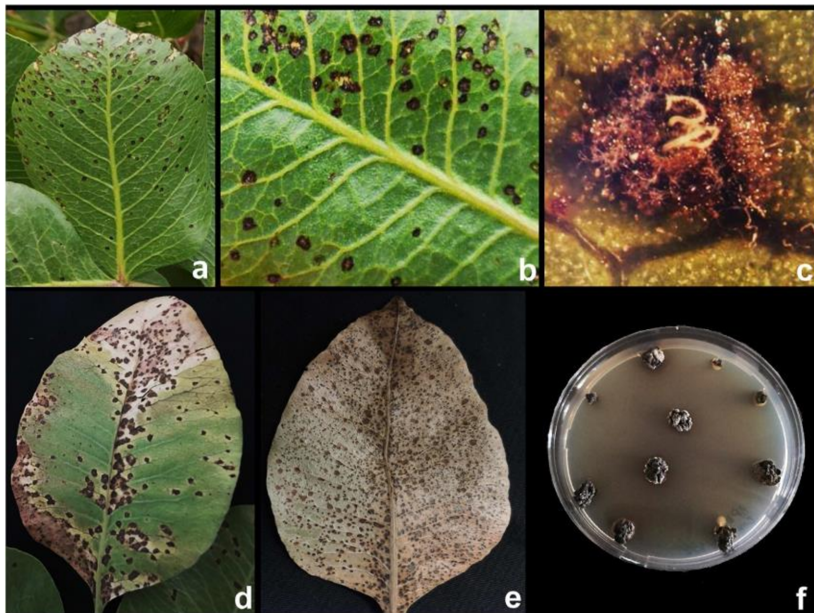


Figure 2. Cultural characteristic of *Septoria pistaciarum*. a-g. 14 days old colony grown on OA (a); SNA (b); CMA (c); WA with stinging nettle (d); MEA (e); PDA gray/black colony (f), red/black colony (g); h, i. Details of two different pigmented isolates grown on PDA.

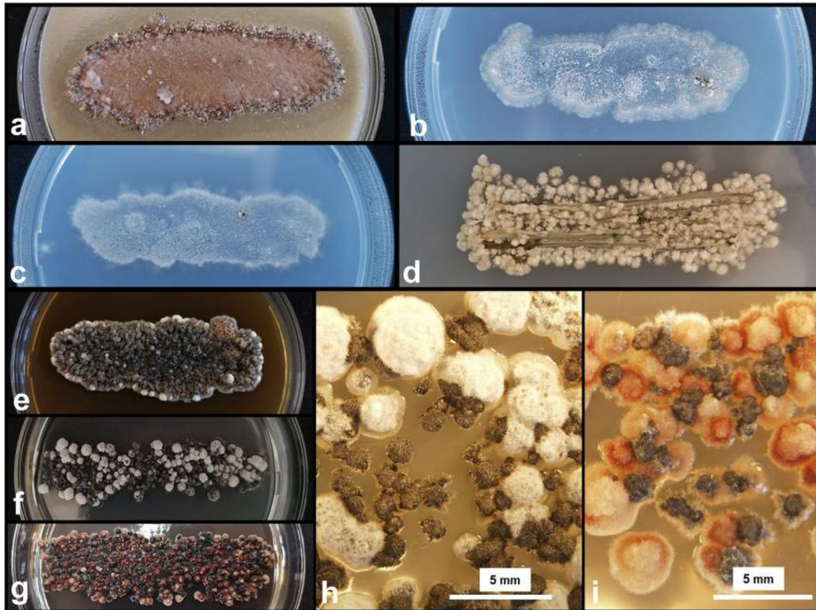


Figure 3. Radial growth rate [mm/day] on different artificial media after 14 days of incubation at 25 ◦C. Letters above the columns indicate treatments significantly different (P < 0.05) according to the LSD test. Vertical bars are the standard errors standard errors.

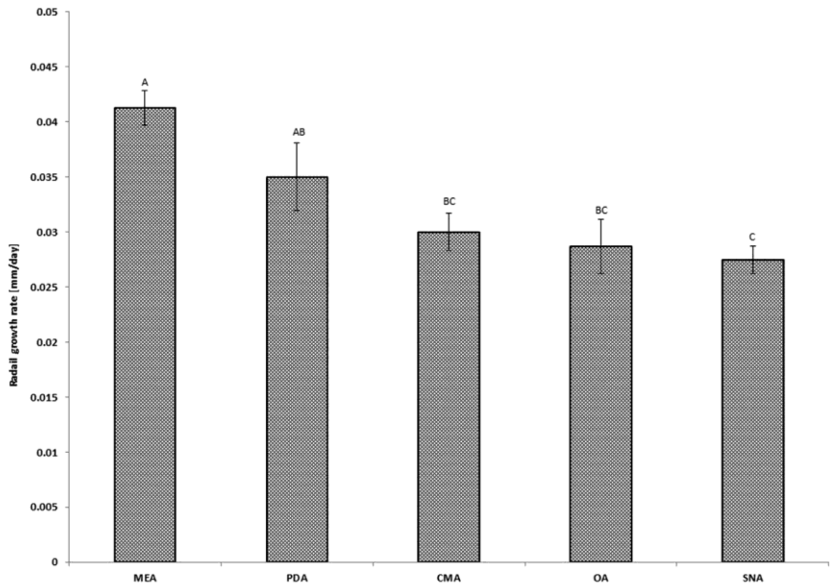


Figure 4. Cultural feature details of *Septoria pistaciarum*. a. Mucilagineous matrix exuded from conidiomata on PDA; b, c. Mucilagineous material from colonies grown on MEA and OA; d, e. Conidia of *S. pistaciarum*; f. Pistachio leaf showing symptoms 23 days after inoculation.

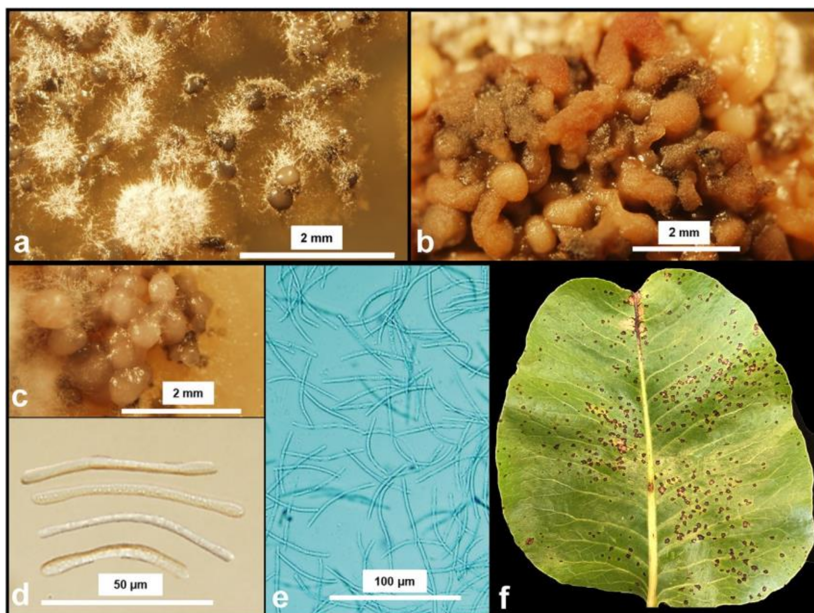
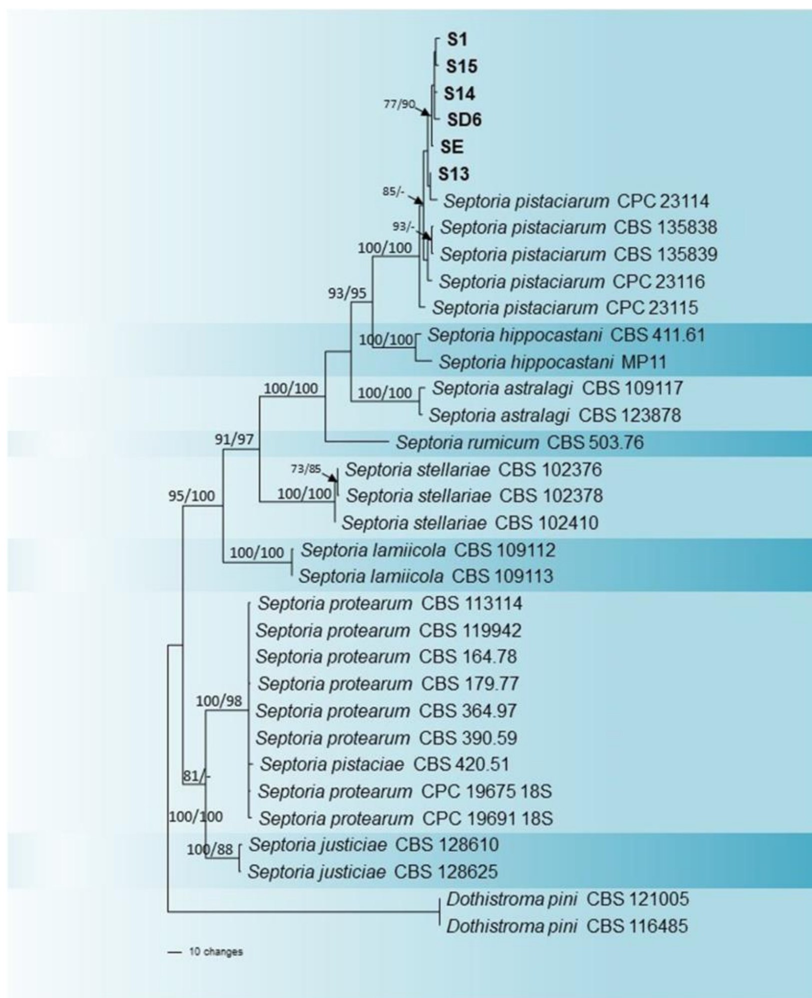


Figure 5. One of 40 equally most parsimonious trees resulting from the analysis of the three-gene combined dataset (ITS + *tef1* + *tub2*) of known *Septoria* species and isolates of this study. Numbers in front and after the slash represent Maximum Parsimony and Maximum Likelihood bootstrap values, respectively. Values represented by "-" were less than 70%. Scale bar represents the number of nucleotide changes.



2.3.2 *DNA amplification and phylogenetic analysis*

For all representative isolates, PCR edited amplicons resulted in 533 bases for partial ITS region, approximately in 522 for *tef1*, and approximately in 446 for *tub2* gene. The preliminary BLASTn search resulted in high identity values (99-100%) with the species confirmed in the phylogenetic tree. The ITS, *tef1*, and *tub2* sequences generated in this study were deposited in GenBank (Table 1). Result of the partition-homogeneity test (P 0.441000) indicates no significant differences in the three genes dataset. The MP analysis of the combined dataset showed that of 1599 total characters (34 taxa), 393 were parsimony-informative, 74 parsimony-uninformative, 1132 were constant. A total of 40 trees were retained. Tree length was 783, CI = 0.775, RI = 0.923, RC = 0.715, and HI = 0.225. For ML analyses, the best-fit model of nucleotide evolution resulted SYM for ITS, GTR+ G for *tef1* and GTR+I for *tub2*. The ML analysis showed that of 1599 total characters, 1132 were constant, 418 parsimony-informative, and 49 autapomorphic. Our isolates strongly clustered with *S. pistaciarum* strains (100/100, MP and ML bootstrap support %, respectively). According to our results, our isolates were identified as *S. pistaciarum* Caracc. 1934 (Fig. 5).

2.3.3 *Pathogenicity test*

The pathogenicity test revealed that *S. pistaciarum* is responsible for causing leaf spot in pistachio. At the first symptoms evaluation, occurring nine days after the inoculation, 28% of the inoculated leaves developed tiny (~1.00 mm^2) characteristic, light brown, rounded spots, still not confined by black margins. In the second evaluation, performed 23 days after inoculation, lesions enlarged and showed characteristic black margins surrounding red to dark brown necrotic spots (Fig. 4f). Lesions ranged from 1.00 to 2.00

mm^2 and 51% of inoculated leaves were infected. In the second evaluation pycnidia were observed in the lesions, isolated and aggregated together as well. Very small lesions (<1.00 mm^2) did not show pycnidia. Controls did not develop lesions. Re-isolations of *S. pistaciarum* from the leaf spots fulfilled the Koch's postulates.

2.4 Discussion

Results of our study confirm, in light of new taxonomic re-classification, that our isolates belong to *S. pistaciarum*, the species described by Caracciolo in 1934 in Sicily (Caracciolo, 1934). Although other authors reported *S. pistaciarum* worldwide (Ahmad *et al.,* 2011; Eskalen *et al.,* 2001; Mass *et al.,* 1971; Young and Michailides, 1989) no molecular data were provided, but identification was done based on only morphological observations and conidia size measurements. For the first time, a study conducted by Crous et al. (2013) elucidated the taxonomic status of septoria-like pathogens associated with pistachio, revealing new taxa classification on the basis of a multi-locus phylogenetic analysis. Results from the study of Crous et al. (2013) led to distinguish *Cylindroseptoria pistaciae*, *Pseudocercospora pistacina* (ex *S. pistacina*), *Septoria pistaciae* and *S. pistaciarum*. *Septoria pistaciae* belongs to the *S. protearum* species complex. Traditionally, the classification of this extensive group of fungi was based on the host association, leading, however, to unreliable identifications. Many Septoria species have broader host range and many species can be found on the same host; reason why the coevolution hypothesis for Septoria is rejected, and the “trans-family host jumping” seems to be the major evolution force-driving for *Septoria* (Quaedvlieg *et al.,* 2013; Verkley *et al.,* 2013). After the first description in 1934 (Caracciolo, 1934), *S. pistaciarum* was denied by Pupillo and Di Caro (1952) who considered it a synonym of *S. pistacina*. Later, Chitzanidis (1956) confirmed the validity of *S. pistaciarum* and described the sexual stage

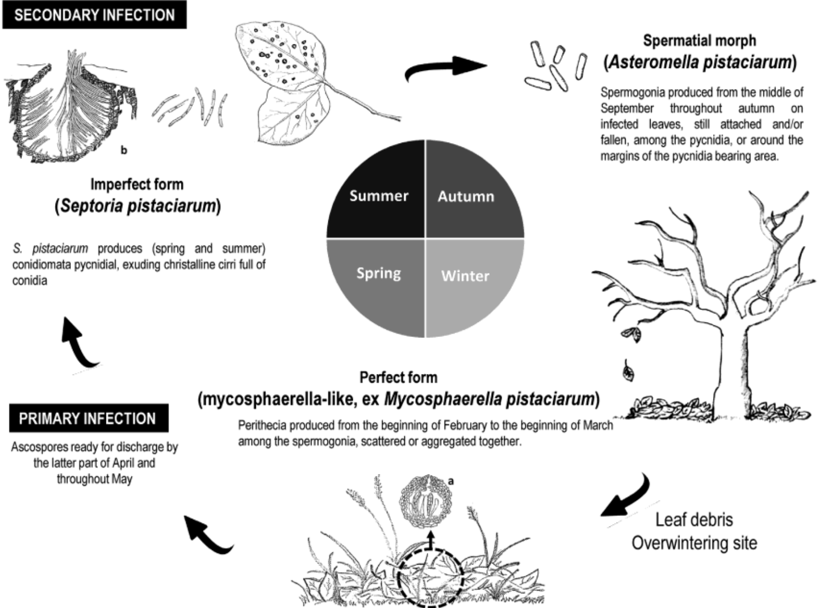
of this species in Greece as *M. pistaciarum*, although, nowadays, the name *Mycosphaerella* referred for the mycosphaerella-like sexual morphs linked to *Septoria* should be avoided (Crous *et al.,* 2013; Quaedvlieg *et al.,* 2013). Results of phylogenetic analysis in this study confirmed that the isolates collected in Sicily strongly grouped into the *S. pistaciarum* clade. Old studies reported also the presence of *S. pistaciae* in Sicily, but only morphological observations were available at that time (Montemartini, 1931; Pupillo and Di Caro, 1952). Crous et al. (2013), provided a taxonomic key (morphology) and our measurements of conidia agree with their description, although *Septoria* specimen collected in the same location, but under different environmental conditions, may result in considerable differences in conidial sizes (Jørstad, 1965). This explains why previous identification, before the molecular era, was particularly difficult and questionable. *S. pistaciarum* was able to grow on each tested media, although best results, in terms of growth and sporulation, was obtained on PDA, MEA and OA. Interestingly, conidia were observed on CMA and SNA, where pycnidia were not observed. Pupillo and Di Caro (1952) erroneously denied *S. pistaciarum*, affirming its synonymy with *S. pistacina*. Describing its morphological characters they noticed hyphal conidia, formed from fertile hypha, and this could explain our observations. Previous studies on many *Septoria* spp. show that shape of conidia on OA generally agree best with those found in the natural substrate (Verkley *et al.,* 2013). Symptoms observed in the field, and replicated in our pathogenicity test, are in accordance with those described by other authors (Caracciolo, 1934; Chitzanidis, 1956). Spots caused by *S. pistaciarum* are easily distinguishable in the field, being more angular and confined by leaf veins (Crous *et al.,* 2013). Our field surveys revealed the presence of leaf spot on *P. vera* as well as on *P. terebinthus*, the latter grown wild and usually used as a rootstock in Sicily, compared to what observed by Young and Michailides (1989), who did not note the disease on *P. atlantica* and *P. terebinthus* leaves.

Although it does not represent the most harmful disease for pistachio production in Sicily, it seems to be the most common and widespread among different production areas of the Island. Observations conducted in Arizona (US) revealed that the onset and severity of the disease were affected by summer rainfall temperatures from 15 to 25 ◦C (Matheron and Call, 1998). Recent investigations in Aegina Island, Greece, showed that the optimum temperature for conidium germination is 23 ◦C with the appearance of first symptoms in the field around mid-May (Thomidis *et al.,* 2021). These results agree with our observations, probably due to the very similar Mediterranean environmental conditions, the island Aegina, Greece and Sicily, Italy. Very few, sporadic and outdated data is available about the epidemiology of this fungus. In this study, conidium was the only reproductive spore observed in the field. Based on our field surveys, and literature available information, we hypothesize *S. pistaciarum* lifecycle characterized by three "states". As observed in this study, and confirmed by other authors (Thomidis *et al.,* 2021), symptoms occur from springtime to the end of summertime with the imperfect form named *S. pistaciarum*, producing conidia from pycnidial conidiomata on the leaf surface. Pycnidiospores germinate on the leaf surface and penetrate through the stomata (Tzavella-Klonari, 1989). Conidia are then dispersed during the season triggering new infections. The second lifecycle state seems to be characterized by the spermatial morph named *Asteromella pistaciarum*, described for the first time in 1947 (Bremer and Petrak, 1947). *Asteromella* was traditionally assigned to those leaf-inhabiting, pycnidia-forming fungi producing tiny rod-shaped, one-celled, hyaline conidia (spermatia) (Ruszkiewicz-Michalska, 2016). However, *Asteromella* classification significantly changed over the years, being defined as a taxonomic entity (von Thümen, 1880), an anamorph genus (Sutton and Hennebert, 1994) and a supposed fertilizing agent in sexual reproduction (andromorph) (Crous, 2009; Parbery, 1996). Chitzanidis (1956) observed spermogonia and spermatia of *A. pistaciarum* in Greece from the middle

of September throughout the autumn, whereas Zachos and Tzavella-Klonari (1971) in December, but nowadays no other recent findings are available to confirm this. In this study, examinations conducted in November on leaves debris showed the presence of empty fruiting bodies similar to pycnidia and/or spermogonia. Since immature pycnidia can be very small and very similar to spermogonia, propagules formation is needed to ascertain the identity of the structure. Moreover, more than one species of leaf-inhabiting fungi produces spermatia within one leaf, and this, certainly, complicates the correct identification (Gerard Verkleij personal communication). In Sicily, the species *L. pistaciae* (ex *L. pistaciae*), recently described, was initially misidentified as *A. pistaciarum* (Vitale *et al.,* 2018). The perfect form (third state) was described by Chitzanidis (1956) as *Mycosphaerella pistaciarum* in Greece. The perfect form is supposedly formed with the andromorphic contribution of *A. pistaciarum*. About the latter, Zachos and Tzavella-Klonari (1971) described in Greece the formation and development of spermogonia and ascocarps of *S. pistaciarum*. The authors observed the presence of spermatia by the tips of trichogynes, this reinforces the assumption of their role as male gametes, although they did not obtain the evidence of fusing and passing of the nucleus into the trichogyne (Zachos and Tzavella-Klonari, 1971). As ascertained for *Sphaerulina musiva* (ex *Septoria musiva*), causal agent of poplar leaf spot and canker, the spermogonial stage was identified in senescent leaves (Luley *et al.,* 1987; Thompson, 1941; Quaedvlieg *et al.,* 2013) and pseudothecia were obtained *in vitro* culturing spermatia with *S. musiva* cultures (Luley *et al.,* 1987). The teleomorph state of *S. pistaciarum* produces ascospores within perithecia from the beginning of February to the beginning of March (Chitzanidis, 1956). Tzavella-Klonari and Zachos (1976) described six distinct phases of the development of perithecia of *M. pistaciarum*, showing how temperatures play a key role in its sexual reproduction. Although the possibility of ascocarps formation exists since early fall, nonoptimal temperatures lead to the ascocarps

degeneration, and only low temperatures (winter) favor their full maturation (Tzavella-Klonari and Zachos, 1976). Ascospores are ready for discharge (wind and rain dispersion) in springtime (April-May) from leaf debris, being the starting point of the primary infection (Fig. 6). The possibility of additional sources of inoculum represented by overwintering mycelia in the tree (i.e., within buds, bark, etc.) should be further investigated. However, due to the lack of molecular data for the spermatial and teleomorphic states in previous literature, the lifecycle of *S. pistaciarum* needs to be entirely investigated and confirmed. Further investigations must be conducted to clarify the epidemiology of this important pathogen of pistachio. The present study represents the first update of Septoria leaf spot on pistachio in Italy since its first description in 1934, and new information is reported here to proceed with additional investigations on an important and widespread disease affecting pistachio in Sicily as well as in many other countries around the world.

Figure 6. Possible cycle of *Septoria pistaciarum*. Seasons details, perithecium with asci (a) and pycnidium with pycnidiospores (b) from Chitzanidis (1956). Illustrations by G. Gusella



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3. Experimental part: Etiology of Botryosphaeria Panicle and Shoot Blight of Pistachio (*Pistacia vera*) caused by *Botryosphaeriaceae* in Italy

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3.1 Introduction

Pistachio (*Pistacia vera*) is an important Mediterranean crop. World top producers are Iran (337,815 tons), United States (335,660), China (106,155), and Turkey (85,000) (www.fao.org). In Italy, pistachio production is centered in the southern regions, with Sicily considered as the first Italian producer. Specifically, the Catania Province is the main producer (3,583.6 t.), followed by Agrigento (236.2 t.), Caltanissetta (202.4 t.), Palermo (37 t.), and Messina (5.5 t.) provinces (http://dati.istat.it). Nowadays, the Bronte area (Catania Province) is the main pistachio growing area in Italy, and its production represents a very important economic resource for the

entire territory (Barone and Marra 2004). Pistachio orchards in this area are defined as "natural pistachio plantings" because they have long been obtained by grafting in situ spontaneous terebinth (*Pistacia terebinthus*) plants naturally spread in the peculiar volcanic soil of the area (Barone et al. 1985). Otherwise, mainly in Agrigento and Caltanissetta provinces, more recent pistachio orchards (from now on: “new” orchards) have a rational design, scheduled irrigation and fertilization, and mechanical harvest (Marino and Marra 2019).

Research over the years showed that several diseases attack pistachios worldwide. The main diseases are represented by Verticillium wilt (*Verticillium dahliae*), Botryosphaeria panicle and shoot blight (*Botryosphaeriaceae* spp.), and Alternaria late blight (*Alternaria alternata*, *A. arborescens, A. infectoria*, and *A. tenuissima* (Eskalen et al. 2001; Michailides et al. 1994; Pryor and Michailides 2002; Yang et al. 2011). Among these diseases, those caused by members of *Botryosphaeriaceae* (*Botryosphaeriales*, *Ascomycota*) represent one of the most serious threat to pistachio production worldwide. The *Botryosphaeriaceae* includes a large group of morphologically diverse fungal species, distributed in all geographical and climatic diversified areas of the world. These fungi are well-known as plant pathogens, endophytes, and saprophytes of woody hosts. Due to their main role as plant pathogens, these fungal species have been studied for a long time, and their impact on forestry and agricultural productions is well known (Phillips et al. 2013). On pistachio species of *Botryosphaeriaceae* can infect many organs such as buds, panicles, fruits, petioles, rachises, mid rib of leaflets, shoots, and branches, leading to fruit panicles killing, hull and kernel decay and dark shell staining, and blight of panicles and shoots (Michailides, 1991; Michailides and Morgan 1992, 2004). The overwintering inoculum is represented by conidia produced within pycnidia (rarely ascospores produced within ascocarps from other hosts) already present within buds, old cankers, rachises, petioles, and fruit mummies (Moral et al. 2019). Due to the high morphological diversity/plasticity

of genera and species of *Botryosphaeriaceae*, the etiology is difficult to estimate without the utility of DNA molecular markers. Following the progress in DNA-based molecular tools, many species have been described associated with pistachio in recent years around the world, including: *Botryosphaeria* dothidea, *B. rhodina, Diplodia seriata, Dothiorella iberica, Dot. sarmentorum, Dot. viticola, Lasiodiplodia americana, L. citricola, L. gilanensis, L. pseudotheobromae, L. theobromae, Macrophomina phaseolina, Neofusicoccum australe, N. hellenicum, N. mediterraneum, N. parvum*, and N. *vitifusiforme* (Armengol et al. 2008; Chen et al. 2014, 2015; Crous et al. 2000; Cunnington et al. 2007; Holevas et al. 2000; Inderbitzin et al. 2010; López-Moral et al. 2020b; Mohammadi et al. 2015; Nouri et al. 2019; Saavedra-Opazo 2011; Sohrabi et al. 2020; Swart and Botes 1995; Wunderlich et al. 2012). Diseases caused by *Botryosphaeriaceae* fungi are defined as monocyclic or oligocyclic (2-3 cycles per season), causing polyetic epidemics (Moral et al. 2019). Thus, a second cycle of secondary infection can occur under the right fall conditions (Michailides, 1991). The progress of epidemics may extend for several years, leading to severe economic losses for growers. In California, in 1998, after a warm and extraordinarily wet spring ("El Niño" year), the disease impact was estimated at 9 million kg of total production losses (Michailides and Morgan 2004; Moral et al. 2019).

In Italy, the phytopathological situation of pistachio has been outdated and poorly investigated, until recent years. The most recent studies of pistachio diseases in Italy reported canker pathogens, such as *Cytospora pistaciae*, *Eutypa lata*, and *Leptosillia pistaciae* (Voglmayr et al) Voglmayr, comb. nov. (ex *Liberomyces pistaciae*) (Aiello et al. 2019; Vitale et al. 2018; Voglmayr et al. 2019). Other recent reports in Italy focused on fruit and foliar pathogens such as *Arthrinium xenocordella, Septoria pistaciarum*, and *Tuberculina persicina* (Aiello et al. 2018; Gusella et al. 2021; Mirabile and Torta 2020). Surveys conducted in Sicily during the growing season of 2019 revealed symptomatic fruit panicles and cankered woody tissues in the

recent orchards in Agrigento and Caltanissetta Provinces. The objectives of this study were to (i) investigate the etiology of the disease by (ii) characterizing the fungal isolates recovered from diseased commercial pistachio orchards based on morphology, optimum growth temperature, and phylogenetic analysis and (iii) testing their pathogenicity.

3.2 Materials And Methods

3.2.1 Field surveys and isolations

Field surveys were conducted during May and August of 2019 in five pistachio orchards located in Agrigento and Caltanissetta Provinces, Sicily, Italy. Orchards surveyed in this study are classified as "new" pistachio plantings (15-30 years old) compared to the traditional "natural" plantings of Bronte area. Symptomatic samples (fruit clusters, leaves, and shoots) were collected and brought to the plant pathology laboratory (Dipartimento di Agricoltura, Alimentazione e Ambiente, Sezione di Patologia vegetale), University of Catania for further investigations. When possible, mummies left hanging on the trees were collected before springtime and observed with Olympus SZX-ILLB2-200 stereoscope (Olympus, Tokyo, Japan). Small sections (0.2 to 0.3 cm^2) of symptomatic tissues (shoots, fruit, leaves, and rachises) 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 sulfate (PDAS; Sigma-Aldrich, St. Louis, MO, USA) to prevent bacterial growth, and then incubated at 25 ± 1 °C for 5 to7 days. Single-hyphal tipped cultures were obtained from pure cultures on PDA and stored in the Plant Pathology Laboratory Collection of the same Department mentioned above. Representative isolates (once

fully characterized) P33, P80, P89, P107, P109 were registered in the CBS Westerdijk Fungal Biodiversity Institute collection (Utrecht) with the following accessions: CBS 147549, CBS 147553, CBS 147552, CBS 147550, CBS 147551 respectively, and used for further investigations. The isolates details are summarized in Table 1.

3.2.2 Morphological and cultural characterization of isolates

To study conidial morphology, sporulation of representative isolates was induced by transferring isolates onto Technical Agar (AT, 1.2% Agar Technical, Biolife, Milan, Italy) with autoclaved pine needles or pistachio leaves (Chen et al. 2014; Smith et al. 1996) placed onto the agar surface, and the plates were incubated at room temperature under UV light. After two weeks, pycnidia were observed with a stereoscope and mounted in lactic acid. A total of 50 conidia were measured (length and width) using an Olympus-BX61 fluorescence microscope (Olympus, Tokyo, Japan). Measurements were captured using the software analySIS 3.2 (Soft Imaging System GmbH, Münster, Germany). Dimensions are reported as the minimum and maximum, and the average in parentheses. To determine the cardinal temperatures of growth, a 5 mm plug of a colony of the isolates mentioned above was removed and transferred to the center of 90-mm Petri dishes of PDA and incubated at 5, 10, 15, 20, 25, 30, 35, and 40°C in the dark. Three Petri dishes were used for each temperature as replicates. The experiment was repeated once. After three days of incubation, the largest and smallest diameters of colonies were measured with a digital scale ruler.

3.2.3 DNA extraction and PCR analysis

Isolates were cultured for 4 days in Potato Dextrose Broth (Microtech scientific, Orange, CA) and mycelium was harvested to extract genomic DNA using the FastDNA Spin kit (MP Biomedicals,

Santa Ana, CA) by following the manufacture's protocol. The final DNA was quantified using NanoDrop 2000C (Thermo Fisher Scientific, Waltham, MA) and diluted to 5 ng/μL with nuclease-free water. The internal transcribed spacer region (ITS) of the nuclear ribosomal RNA cluster was amplified with primers ITS5 and ITS4 (White et al. 1990). Part of the translation elongation factor 1α gene (*tef1-α*) was amplified using primers EF1-728F and EF1-986R (Carbone and Kohn, 1999), while the primer set Bt2a and Bt2b (Glass and Donaldson, 1995) was used for the partial β-tubulin gene (*tub2*). Regular PCR was conducted for ITS and *tub2* according to Luo et al. (2017). ITS PCR amplification conditions were set as follows: initial denaturation temperature of 95°C for 4 min, followed by 35 cycles at the denaturation temperature of 94°C for 30 s, annealing temperature of 58°C for 30 s, extension at 72°C for 45 s and final extension at 72°C for 7 min. *Tub2* conditions were: initial denaturation temperature of 94°C for 2 min, followed by 35 cycles at the denaturation temperature of 94°C for 30 s, annealing temperature of 56°C for 45 s, extension at 72°C for 30 s and final extension at 72°C for 7 min. All PCR amplicons were separated on 1.5% agarose gels in Tris-acetate buffer (90 V for 35 min) stained with GelRed® (Biotium, Fremont, CA) to determine the existence of the expected PCR product. *Tef1-α* region was amplified using qPCR according to Luo et al. (2017). The following parameters were used for qPCR amplifications: an initial preheat for 4 min at 95°C, followed by 40 cycles at 95°C for 30 s, 57°C for 30 s, 72°C for 30 s and then 72°C for 5 min. PCR products were purified using ExoSAP-IT™ (USB, Cleveland, OH, USA) and stored at -20°C. Resulting amplicons were sequenced in both directions. Sequence reactions were run by using an automated sequencer by the Division of Biological Sciences sequencing facility, University of California-Davis.

3.2.4 Phylogenetic analysis

Sequences were read and edited using MEGAX: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar et al. 2018) and Mesquite v. 3.61 (Maddison and Maddison 2019). 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 Mesquite v. 3.10, and manual alignments were made when necessary. A partition-homogeneity test with heuristic search and 1,000 homogeneity replicates was performed using PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0a (Swofford, 2003) to test for discrepancies among the three gene dataset. For comparison, 39 additional sequences were selected according to the taxonomic classification on the *Botryosphaeriaceae* genera and species provided by Phillips et al. (2013), Zhang et al. (2021), and previous works on *Botryosphaeriaceae* affecting pistachio (Chen et al. 2014, 2015; López-Moral et al. 2020b) 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 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 1,000 bootstrap replicates were performed to test the robustness of the tree topologies. MrModeltest v. 2.4 (Nylander, 2004) was used to identify the best-fit model of nucleotide evolution for each gene according to the Akaike information criterion (AIC). The maximum likelihood analysis (ML) of the combined genes was performed in GARLI v.0.951 (Zwickl, 2006), and clade support was assessed by 1,000 bootstrap replicates. *Guignardia philoprina* (CBS 447.68) and *Phyllosticta citricarpa* (CBS 102374) served as the outgroup in both analyses.

3.2.5 *Pathogenicity test on detached shoots*

A preliminary pathogenicity test on detached pistachio shoots was conducted under laboratory conditions to determine the ability to cause cankers by the representative isolates. One-year-old lignified shoots of pistachio cv. Bianca were collected from mature trees growing in Bronte (Catania Province) orchards. The shoots were cut to 30 to 35 cm segments, submerged for 4 min in 0.5% of household bleach (3.5% NaOCl), and rinsed twice with sterile distilled water before inoculation. The outer bark of shoots (six shoots per fungal isolate and two wounds per shoot) was removed using a 5-mm cork borer, and a mycelial plug of *B. dothidea* (P89), *N. hellenicum* (P109), and *N. mediterraneum* (P107), was inserted into the wounds upside down and sealed with Parafilm® to prevent desiccation. Inoculations with sterile PDA plugs served as negative controls. Inoculated shoots were placed in plastic containers (60 × 39 × 6 cm) and then incubated in a growth chamber with a 12 h photoperiod at 25°C ± 1°C for 21 days. Presence of wood discoloration, and presence of pycnidia on the inoculated surface were recorded. Re-isolations were made from the margin of developing cankers to confirm the identity of the pathogens as described before.

3.2.6 *Pathogenicity test in the field*

Pathogenicity tests in the field were conducted in an experimental pistachio orchard of the University of Catania in June and July 2021. Four trees cv. Bianca grafted on terebinth for each fungal species were selected and three two-year-old shoots in each pistachio tree were used. Inoculations were conducted by using a 5-mm mycelium plug from a 7-day-old culture of *B. dothidea* (P80), *N. hellenicum* (P109), and *N. mediterraneum* (P107). The inoculation site was first surface disinfected by spraying with 70% ethanol solution,

and wounds were made with a sterilized 5-mm cork borer to remove the bark, and a mycelium plug was placed upside down into the plant tissue wound. Wounds were sealed with Parafilm®. A total of 12 additional shoots inoculated with sterile PDA plugs served as controls. Lesion lengths were measured 39 days after the inoculations. Re-isolations were conducted as described above. Moreover, the same fungal isolates were inoculated on fruit clusters in the same experimental orchard. A total of 30 fruits (ten fruits per tree) were randomly chosen and wounded with a 4-mm cork borer by removing a piece of hull and applying 4-mm mycelium plugs from a 6-days-old cultures onto the wounds. Controls consisted of fruits inoculated with water agar plugs. Measurements (two perpendicular diameters) of the lesions were conducted five days after the inoculations. Re-isolations were performed as described above.

3.2.7 Data analysis

Dataset of optimal mycelial growth rate and lesion length measurements of pathogenicity tests were analyzed in Statistix 10 (Analytical Software 2013). In the optimal mycelial growth rate experiment, repetitions data were tested for homogeneity of variances using the Levene's Test, and combined in one dataset The growth data were transformed to radial growth rate (mm day-1). A nonlinear adjustment of the dataset (mean values for each temperature) was applied via the generalized Analytis β model, using the equation described by Moral et al. (2012), and the optimum growth temperature was calculated according to the formula provided by the same authors. For pathogenicity tests the analysis of variance (ANOVA) of lesion length measurements on shoots, as well as on fruits was performed and mean differences were compared with the Fisher's protected Least Significance Difference (LSD) Test at $P = 0.05$.

Table 1. Information of fungal isolates used in the phylogenetic analysis and their corresponding GenBank accession numbers. Isolates in bold are from this study.

Species	Isolate	Host/Culitvar	Location	Collector	GenBank accession no.		
					ITS	*tef1-α*	*tub2*
Botryosphaeria corticis	CBS 119047	*Vaccinium corymbosum*	New Jersey, USA	P.V. Oudemans	DQ299245	EU017539	EU673107
Botryosphaeria corticis	ATCC 22927	*Vaccinium* sp.	North Carolina, USA	R.D. Millholland	DQ299247	EU673291	EU673108
Botryosphaeria dothidea	CMW8000	*Prunus* sp.	Crocifisso, Switzerland	B. Slippers	AY236949	AY236898	AY236927
Botryosphaeria dothidea	CBS 110302	*Vitis vinifera*	Montemor-o-Novo, Portugal	A. J. L. Phillips	AY259092	AY573218	EU673106
Botryosphaeria dothidea	Colpat-462	*Pistacia vera* cv. Aguilar	Archidona, Málaga, Spain	-	MN654324	MN706174	MN794197
Botryosphaeria dothidea	2A06	*Pistacia vera*	Winters, Yolo, CA, USA	-	KF955667	KF955766	KF955865
Botryosphaeria dothidea	3H48	*Pistacia vera*	Kern, CA, USA	-	KF955674	KF955773	KF955872
Botryosphaeria dothidea	4D15	*Pistacia vera*	Imathias, Greece	-	KF955677	KF955776	KF955875
Botryosphaeria dothidea	1E09.	*Pistacia vera*	Thessaloniki, Greece	-	KF955678	KF955777	KF955876
Botryosphaeria dothidea	**P80**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350233**	**MZ358193**	**MZ358258**
Botryosphaeria dothidea	**P85**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350234**	**MZ358194**	**MZ358259**
Botryosphaeria dothidea	**P87**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350235**	**MZ358195**	**MZ358260**

Botryosphaeria dothidea	**P88**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350236**	**MZ358196**	**MZ358261**
Botryosphaeria dothidea	**P89**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350237**	**MZ358197**	**MZ358262**
Botryosphaeria dothidea	**P90**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350238**	**MZ358198**	**MZ358263**
Guignardia philoprina	CBS 447.68	*Taxus baccata*	Netherlands	H.A. van der Aa	FJ824768	FJ824773	FJ824779
Neofusicoccum cordaticola	CBS 123634	*Syzygium cordatum*	Sodwana Bay, South Africa	D. Pavlic	EU821898	EU821868	EU821838
Neofusicoccum cordaticola	CBS 123635	*Syzigium cordatum*	South Africa	D. Pavlic	EU821903	EU821873	EU821843
Neofusicoccum hellenicum	CERC 1947	*Pistacia vera*	Thessaloniki, Greece	T.J. Michailides	KP217053	KP217061	KP217069
Neofusicoccum hellenicum	CERC 1948	*Pistacia vera*	Aghios Mamas, Chalkidiki, Greece	T.J. Michailides	KP217054	KP217062	KP217070
Neofusicoccum hellenicum	CERC 1953	*Pistacia vera*	Aghios Mamas, Chalkidiki, Greece	T.J. Michailides	KP217055	KP217063	KP217071
Neofusicoccum hellenicum	CERC 1957	*Pistacia vera*	Imathias, Greece	T.J. Michailides	KP217056	KP217064	KP217072
Neofusicoccum hellenicum	CERC 1959	*Pistacia vera*	Imathias, Greece	T.J. Michailides	KP217057	KP217065	KP217073
Neofusicoccum hellenicum	**P109**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350239**	**MZ358199**	**MZ358264**
Neofusicoccum kwambonambiense	CBS 123639	*Syzigium cordatum*	South Africa	D. Pavlic	EU821900	EU821870	EU821840
Neofusicoccum kwambonambiense	CBS 123641	*Syzigium cordatum*	South Africa	D. Pavlic	EU821919	EU821889	EU821859
Neofusicoccum mediterraneum	CBS 121718	*Eucalyptus* sp.	Greece	P.W. Crous, M.J. WingfieldA.J.L. Phillips	GU251176	GU251308	GU251836
Neofusicoccum mediterraneum	CBS 121558	*Olea europea*	Italy	F. Salvatore	GU799463	GU799462	GU799461
Neofusicoccum mediterraneum	Colpat-454	*Pistacia vera* cv. Kerman	Puente Genil, Córdoba, Spain	-	MN654314	MN706161	MN839590

Neofusicoccum mediterraneum	Colpat-461	*Pistacia vera* cv. Aguilar	Archidona, Málaga, Spain	-	MN654315	MN706168	MN839591
Neofusicoccum mediterraneum	1F77	*Pistacia vera*	Parlier, Fresno, CA, USA	-	KF955746	KF955845	KF955944
Neofusicoccum mediterraneum	2F03	*Pistacia vera*	Chico, Butte, CA, USA	-	KF955698	KF955797	KF955896
Neofusicoccum mediterraneum	2K10	*Pistacia vera*	Chico, Butte, CA, USA	-	KF955714	KF955813	KF955912
Neofusicoccum mediterraneum	3C32	*Pistacia vera*	Chico, Butte, CA, USA	-	KF955732	KF955831	KF955930
Neofusicoccum mediterraneum	**P21**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350240**	**MZ358200**	**MZ358265**
Neofusicoccum mediterraneum	**P22**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350241**	**MZ358201**	**MZ358266**
Neofusicoccum mediterraneum	**P23**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350242**	**MZ358202**	**MZ358267**
Neofusicoccum mediterraneum	**P25**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350243**	**MZ358203**	**MZ358268**
Neofusicoccum mediterraneum	**P27**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350244**	**MZ358204**	**MZ358269**
Neofusicoccum mediterraneum	**P28**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350245**	**MZ358205**	**MZ358270**
Neofusicoccum mediterraneum	**P29**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350246**	**MZ358206**	**MZ358271**
Neofusicoccum mediterraneum	**P30**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350247**	**MZ358207**	**MZ358272**
Neofusicoccum mediterraneum	**P32**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350248**	**MZ358208**	**MZ358273**
Neofusicoccum mediterraneum	**P33**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350249**	**MZ358209**	**MZ358274**
Neofusicoccum mediterraneum	**P37**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350250**	**MZ358210**	**MZ358275**
Neofusicoccum mediterraneum	**P38**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350251**	**MZ358211**	**MZ358276**
Neofusicoccum mediterraneum	**P40**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350252**	**MZ358212**	**MZ358277**

Neofusicoccum mediterraneum	**P41**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350253**	**MZ358213**	**MZ358278**
Neofusicoccum mediterraneum	**P43**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350254**	**MZ358214**	**MZ358279**
Neofusicoccum mediterraneum	**P44**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350255**	**MZ358215**	**MZ358280**
Neofusicoccum mediterraneum	**P46**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350256**	**MZ358216**	**MZ358281**
Neofusicoccum mediterraneum	**P50**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350257**	**MZ358217**	**MZ358282**
Neofusicoccum mediterraneum	**P63**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350258**	**MZ358218**	**MZ358283**
Neofusicoccum mediterraneum	**P64**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350259**	**MZ358219**	**MZ358284**
Neofusicoccum mediterraneum	**P65**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350260**	**MZ358220**	**MZ358285**
Neofusicoccum mediterraneum	**P66**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350261**	**MZ358221**	**MZ358286**
Neofusicoccum mediterraneum	**P67**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350262**	**MZ358222**	**MZ358287**
Neofusicoccum mediterraneum	**P68**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350263**	**MZ358223**	**MZ358288**
Neofusicoccum mediterraneum	**P69**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350264**	**MZ358224**	**MZ358289**
Neofusicoccum mediterraneum	**P70**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350265**	**MZ358225**	**MZ358290**
Neofusicoccum mediterraneum	**P71**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350266**	**MZ358226**	**MZ358291**
Neofusicoccum mediterraneum	**P73**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350267**	**MZ358227**	**MZ358292**
Neofusicoccum mediterraneum	**P74**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350268**	**MZ358228**	**MZ358293**
Neofusicoccum mediterraneum	**P75**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350269**	**MZ358229**	**MZ358294**
Neofusicoccum mediterraneum	**P76**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350270**	**MZ358230**	**MZ358295**
Neofusicoccum mediterraneum	**P77**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350271**	**MZ358231**	**MZ358296**

Neofusicoccum mediterraneum	**P79**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350272**	**MZ358232**	**MZ358297**
Neofusicoccum mediterraneum	**P92**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350273**	**MZ358233**	**MZ358298**
Neofusicoccum mediterraneum	**P93**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350274**	**MZ358234**	**MZ358299**
Neofusicoccum mediterraneum	**P94**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350275**	**MZ358235**	**MZ358300**
Neofusicoccum mediterraneum	**P95**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350276**	**MZ358236**	**MZ358301**
Neofusicoccum mediterraneum	**P97**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350277**	**MZ358237**	**MZ358302**
Neofusicoccum mediterraneum	**P98**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350278**	**MZ358238**	**MZ358303**
Neofusicoccum mediterraneum	**P99**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350279**	**MZ358239**	**MZ358304**
Neofusicoccum mediterraneum	**P101**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350280**	**MZ358240**	**MZ358305**
Neofusicoccum mediterraneum	**P102**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350281**	**MZ358241**	**MZ358306**
Neofusicoccum mediterraneum	**P103**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350282**	**MZ358242**	**MZ358307**
Neofusicoccum mediterraneum	**P104**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350283**	**MZ358243**	**MZ358308**
Neofusicoccum mediterraneum	**P105**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350284**	**MZ358244**	**MZ358309**
Neofusicoccum mediterraneum	**P107**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350285**	**MZ358245**	**MZ358310**
Neofusicoccum mediterraneum	**P108**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350286**	**MZ358246**	**MZ358311**
Neofusicoccum mediterraneum	**P110**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350287**	**MZ358247**	**MZ358312**
Neofusicoccum mediterraneum	**P111**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350288**	**MZ358248**	**MZ358313**
Neofusicoccum mediterraneum	**P112**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350289**	**MZ358249**	**MZ358314**
Neofusicoccum mediterraneum	**P113**	*Pistacia vera*	**Sicily, Italy**	**G. Gusella**	**MZ350290**	**MZ358250**	**MZ358315**

Neofusicoccum mediterraneum	**P115**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350291**	**MZ358251**	**MZ358316**
Neofusicoccum mediterraneum	**P116**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350292**	**MZ358252**	**MZ358317**
Neofusicoccum mediterraneum	**P118**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350293**	**MZ358253**	**MZ358318**
Neofusicoccum mediterraneum	**P119**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350294**	**MZ358254**	**MZ358319**
Neofusicoccum mediterraneum	**P120**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350295**	**MZ358255**	**MZ358320**
Neofusicoccum mediterraneum	**P122**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350296**	**MZ358256**	**MZ358321**
Neofusicoccum mediterraneum	**P123**	***Pistacia vera***	**Sicily, Italy**	**G. Gusella**	**MZ350297**	**MZ358257**	**MZ358322**
Neofusicoccum occulatum	CBS 128008	*Eucalyptus grandis* hybrid	Australia	T.I. Burgess	EU301030	EU339509	EU339472
Neofusicoccum occulatum	MUCC286	*Eucalyptus pellita*	Australia	T.I. Burgess	EU736947	EU339511	EU339474
Neofusicoccum parvum	CMW9081	*Populus nigra*	New Zealand	G.J. Samuels	AY236943	AY236888	AY236917
Neofusicoccum parvum	CBS 110301	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259098	AY573221	EU673095
Neofusicoccum ribis	CBS 115475	*Ribes* sp.	USA	B. Slippers & G. Hudler	AY236935	AY236877	AY236906
Neofusicoccum ribis	CBS 124924	*Terminalia catappa*	Cameroon	D. Begoude & J. Roux	FJ900607	FJ900653	FJ900634
Neofusicoccum ribis	CBS 124923	*Terminalia catappa*	Cameroon	D. Begoude & J. Roux	FJ900608	FJ900654	FJ900635
Neofusicoccum ribis	CBS 123645	*Syzigium cordatum*	South Africa	D. Pavlic	EU821904	EU821874	EU821844
Neofusicoccum ribis	CBS 123646	*Syzigium cordatum*	South Africa	D. Pavlic	EU821905	EU821875	EU821845
Neofusicoccum viticlavatum	CBS 112878	*Vitis vinifera*	South Africa	F. Halleen	AY343381	AY343342	KX465058
Neofusicoccum vitifusiforme	CBS 110887	*Vitis. Vinifera*	South Africa	J.M. van Niekerk	AY343383	AY343343	KX465061

Phyllosticta citricarpa	CBS 102374	*Citrus aurantium*	Brazil	-	FJ824767	FJ538371	FJ824778

Table 2. Isolates of *Botryosphaeriaceae* collected from Agrigento and Caltanissetta provinces of Sicily in this study.

Species	Isolate	Location	Farm
Botryosphaeria dothidea	**P80; CBS 147553**	Agrigento	1
Botryosphaeria dothidea	**P85**	Agrigento	1
Botryosphaeria dothidea	**P87**	Agrigento	2
Botryosphaeria dothidea	**P88**	Agrigento	2
Botryosphaeria dothidea	**P89; CBS 147552**	Agrigento	2
Botryosphaeria dothidea	**P90**	Agrigento	2
Neofusicoccum hellenicum	**P109; CBS 147551**	Agrigento	3
Neofusicoccum mediterraneum	**P21**	Caltanissetta	5
Neofusicoccum mediterraneum	**P22**	Caltanissetta	5
Neofusicoccum mediterraneum	**P23**	Caltanissetta	5
Neofusicoccum mediterraneum	**P25**	Caltanissetta	5
Neofusicoccum mediterraneum	**P27**	Caltanissetta	5
Neofusicoccum mediterraneum	**P28**	Caltanissetta	5
Neofusicoccum mediterraneum	**P29**	Caltanissetta	5
Neofusicoccum mediterraneum	**P30**	Caltanissetta	5
Neofusicoccum mediterraneum	**P32**	Caltanissetta	5
Neofusicoccum mediterraneum	**P33; CBS 147549**	Caltanissetta	5
Neofusicoccum mediterraneum	**P37**	Caltanissetta	5
Neofusicoccum mediterraneum	**P38**	Caltanissetta	5
Neofusicoccum mediterraneum	**P40**	Caltanissetta	5
Neofusicoccum mediterraneum	**P41**	Caltanissetta	5
Neofusicoccum mediterraneum	**P43**	Caltanissetta	5
Neofusicoccum mediterraneum	**P44**	Caltanissetta	5
Neofusicoccum mediterraneum	**P46**	Caltanissetta	5
Neofusicoccum mediterraneum	**P50**	Caltanissetta	5
Neofusicoccum mediterraneum	**P63**	Agrigento	3
Neofusicoccum mediterraneum	**P64**	Agrigento	3

Neofusicoccum mediterraneum	**P65**	Agrigento	3
Neofusicoccum mediterraneum	**P66**	Agrigento	3
Neofusicoccum mediterraneum	**P67**	Agrigento	3
Neofusicoccum mediterraneum	**P68**	Agrigento	3
Neofusicoccum mediterraneum	**P69**	Agrigento	3
Neofusicoccum mediterraneum	**P70**	Agrigento	3
Neofusicoccum mediterraneum	**P71**	Agrigento	3
Neofusicoccum mediterraneum	**P73**	Agrigento	3
Neofusicoccum mediterraneum	**P74**	Agrigento	3
Neofusicoccum mediterraneum	**P75**	Agrigento	3
Neofusicoccum mediterraneum	**P76**	Agrigento	3
Neofusicoccum mediterraneum	**P77**	Agrigento	3
Neofusicoccum mediterraneum	**P79**	Agrigento	1
Neofusicoccum mediterraneum	**P92**	Agrigento	3
Neofusicoccum mediterraneum	**P93**	Agrigento	3
Neofusicoccum mediterraneum	**P94**	Agrigento	3
Neofusicoccum mediterraneum	**P95**	Agrigento	3
Neofusicoccum mediterraneum	**P97**	Agrigento	3
Neofusicoccum mediterraneum	**P98**	Agrigento	3
Neofusicoccum mediterraneum	**P99**	Agrigento	3
Neofusicoccum mediterraneum	**P101**	Agrigento	3
Neofusicoccum mediterraneum	**P102**	Agrigento	3
Neofusicoccum mediterraneum	**P103**	Agrigento	3
Neofusicoccum mediterraneum	**P104**	Agrigento	3
Neofusicoccum mediterraneum	**P105**	Agrigento	3
Neofusicoccum mediterraneum	**P106**	Agrigento	3
Neofusicoccum mediterraneum	**P107; CBS 147550**	Agrigento	3
Neofusicoccum mediterraneum	**P108**	Agrigento	3
Neofusicoccum mediterraneum	**P110**	Agrigento	3
Neofusicoccum mediterraneum	**P111**	Agrigento	3
Neofusicoccum mediterraneum	**P112**	Agrigento	3
Neofusicoccum mediterraneum	**P113**	Agrigento	3
Neofusicoccum mediterraneum	**P115**	Agrigento	3

Neofusicoccum mediterraneum	**P116**	Agrigento	4
Neofusicoccum mediterraneum	**P118**	Agrigento	4
Neofusicoccum mediterraneum	**P119**	Agrigento	4
Neofusicoccum mediterraneum	**P120**	Agrigento	4
Neofusicoccum mediterraneum	**P122**	Agrigento	4
Neofusicoccum mediterraneum	**P123**	Agrigento	4

*3.3 **Results***

3.3.1 Field surveys and isolations

Symptoms observed in the field included shoot dieback, wood discoloration (i.e., necrotic and sunken lesion on lignified tissue), sometimes evolving in external cankers, and rachis black discoloration (Fig. 1a-d). Lesions on fruit epicarp appeared as black rounded spots, enlarging with time (Fig. 1e, f), whereas lesions on leaf surface were large, necrotic, and coalescing, with no defined margins (Fig. 1g). Symptoms observed in the field are summarized in Table 2. In addition, field surveys conducted at the end of February often revealed

the nut mummies hanging from the trees. Closer observation of mummified nuts sometimes revealed a characteristic silver-gray color, and numerous pycnidia, typical of *Botryosphaeriaceae*/*Diaporthaceae* colonization during the previous seasons (Fig. 2a-b). All the symptomatic tissues consistently yielded *Botryosphaeriaceae*-like fungi, characterized, as reported by Slippers and Wingfield (2007), by a 'fluffy' mycelium, either white to creamy or pigmented 'greenish brown' or grey to grey black (Fig. 2c). Results of the isolations showed *Botryosphaeriaceae*-like fungi from fruit spots (89.5%), leaf lesions (98.8%), rachis discoloration (36.9%), shoot cankers (38.9%), and shoot dieback (36.9%). Leaf lesions observed during field surveys were very similar to late infections lesions of *S. pistaciarum*. Probably, both infections occurred in the leaves and *Botryosphaeriaceae*, due to the faster mycelial growth, appeared first from the isolations.

3.3.2 Morphological and cultural characterization of the isolates

The species *B. dothidea* cultured on PDA showed fluffy aerial mycelia, smoke grey to olivaceous (Fig. 2d). Conidia of isolate P85 measured: 14.3 – (21.1) – 27.1 × 3.7 – (5.6) –7.4 µm. After 3 days of incubation, no mycelial growth was observed at 5, 10, and 40°C for both isolates of *B. dothidea*. The highest growth rate for P89 (12.0 mm day-1) was observed at 30° C, whereas 12.8 mm day-1 for isolate P80 at 25°C. According to the Analytis β model, the optimal growth temperature for both isolates resulted at 30.8 and 30.0°C, respectively. *Neofusicoccum hellenicum* isolate P109 cultured on PDA was characterized by cottony aerial mycelium, initially white, turning mouse grey with time (Fig. 2e). Conidia measured 14.8 – (21.5) – 26.0 × 4.4 – (5.8) – 7.2 µm. *Neofusicoccum hellenicum* did not grow at 5 and 40°C, showing the highest growth rate (12.1 mm day-1) at 25°C, and the optimal growth temperature resulted at 29.9°C. *Neofusicoccum mediterraneum* isolates P33 and P107 cultured on

PDA showed grey to olive- green colonies adherent to the medium, turning dark grey with time (Fig. 2f). Conidia of isolate P107 were 13.7 – (20.0) – 26.2 × 4.4 – (5.8) – 6.6 μm. Isolates P33 and P107 did not grow at 5 and 40°C, but grew fastest (growth rate 5.2 and 6.9-mm day-1, respectively) at 25°C. The optimal growth temperature for both isolates was 26.8°C. All results of the effect of temperature on mycelium growth rate are shown in Fig. 3.

Figure 1. Symptoms of *Botryosphaeriaceae* observed in the field. a, Shoot dieback in the middle of the green canopy. b-c, Internal wood discoloration (shoot canker). d, Rachis infection. The infection started from the rachis and caused the blight of the nuts. e-f Fruit lesion (spot) on the epicarp. g, Leaf lesions.

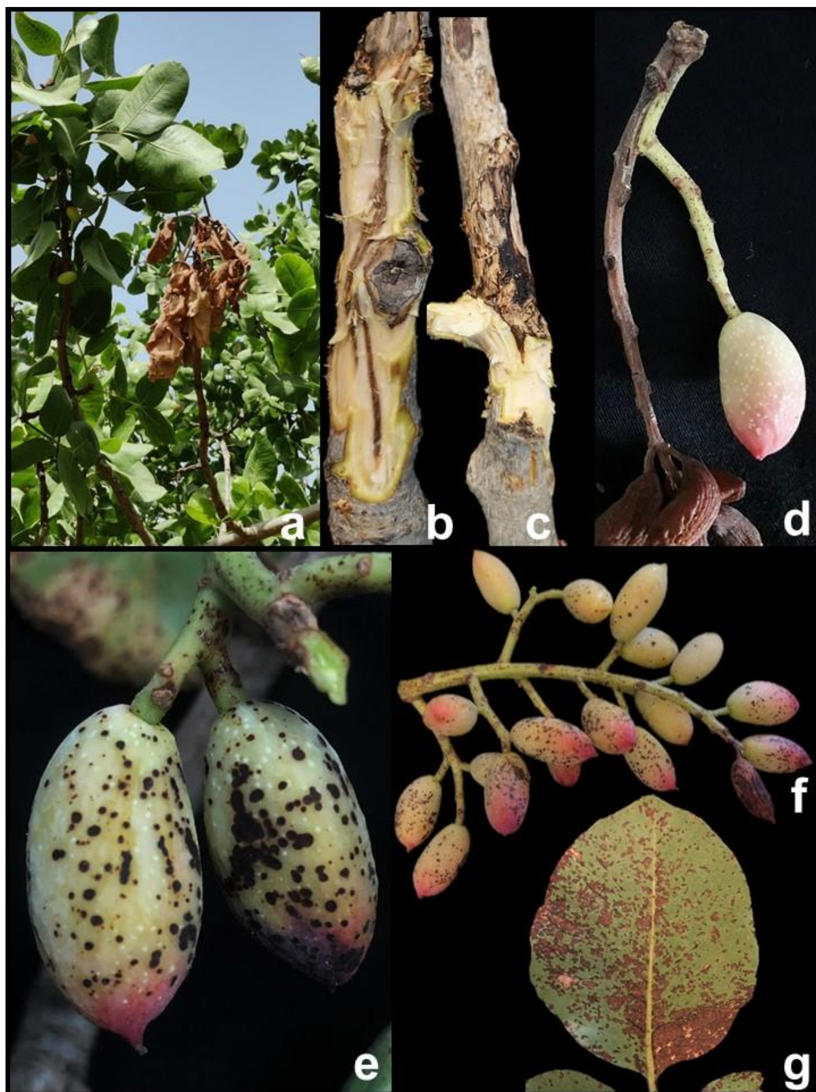

Figure 2. a Silver grey mummies hanging from the tree because of retained rachises. b, Mummies showing numerous pycnidia developed in the epicarp. c, Botryosphaeriaceae colonies from isolations. d, *Botryosphaeria dothidea* cultured on PDA at 7 days (left) and 14 (right). e, *Neofusicoccum hellenicum* cultured on PDA, 7 days (left), 14 days (right). f, *Neofusicoccum mediterraneum* cultured on PDA, 7 days (left), 14 days (right).

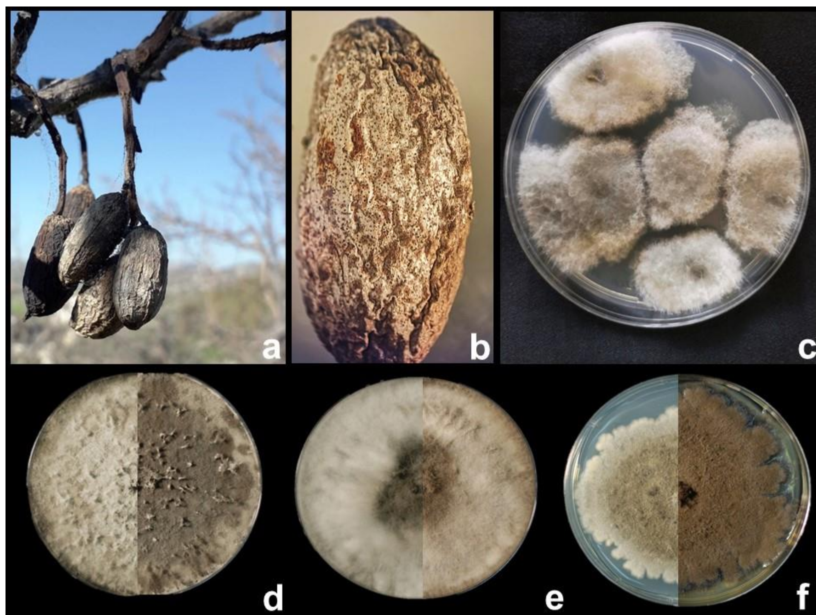

Figure 3. Effect of temperature on mycelial growth rate of Botryosphaeriaceae spp. isolated from pistachios in Sicily. The averages of radial growth rate, and temperatures were adjusted to a nonlinear regression curve via the Analytis β model. Data points are the means of two independent experiments of three replicated Petri dishes. Vertical bars are the standard error of the means.

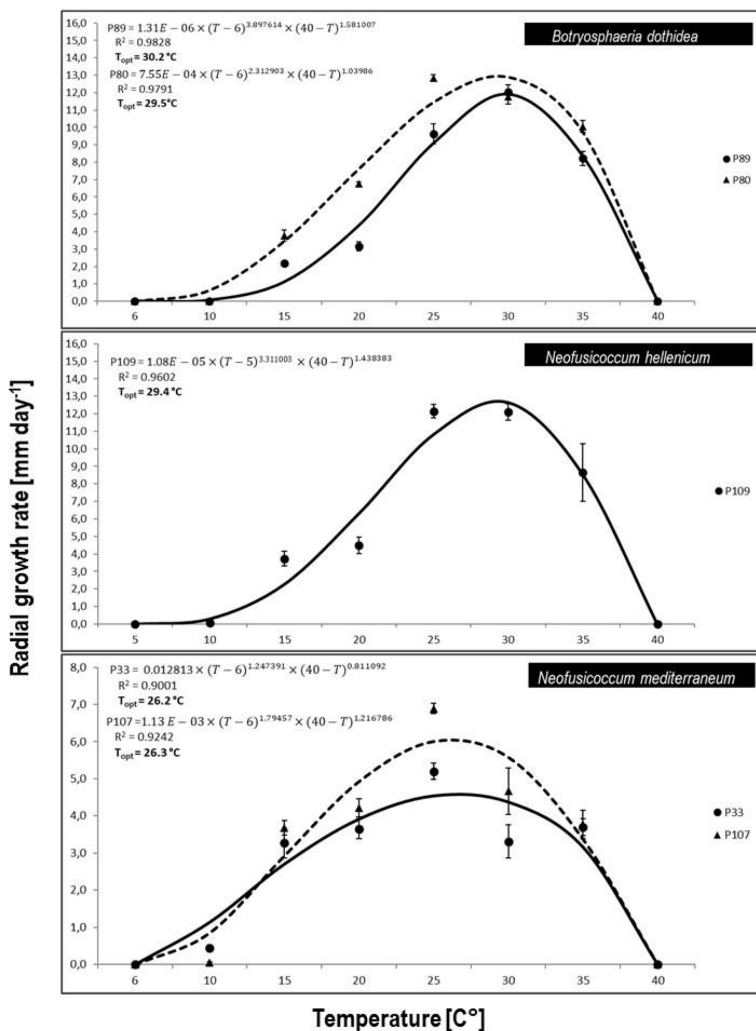

Figure 4. One of 47 equally most parsimonious trees generated from maximum parsimony analysis of the three-gene (ITS + *tef1-α*+ *tub2*) combined dataset from Botryosphaeriaceae species. Numbers in front and after the slash represent parsimony and likelihood bootstrap values from 1,000 replicates, respectively. Letter 'T' indicates type material. Isolates in bold are generated in this study. Bar indicates the number of nucleotide changes.

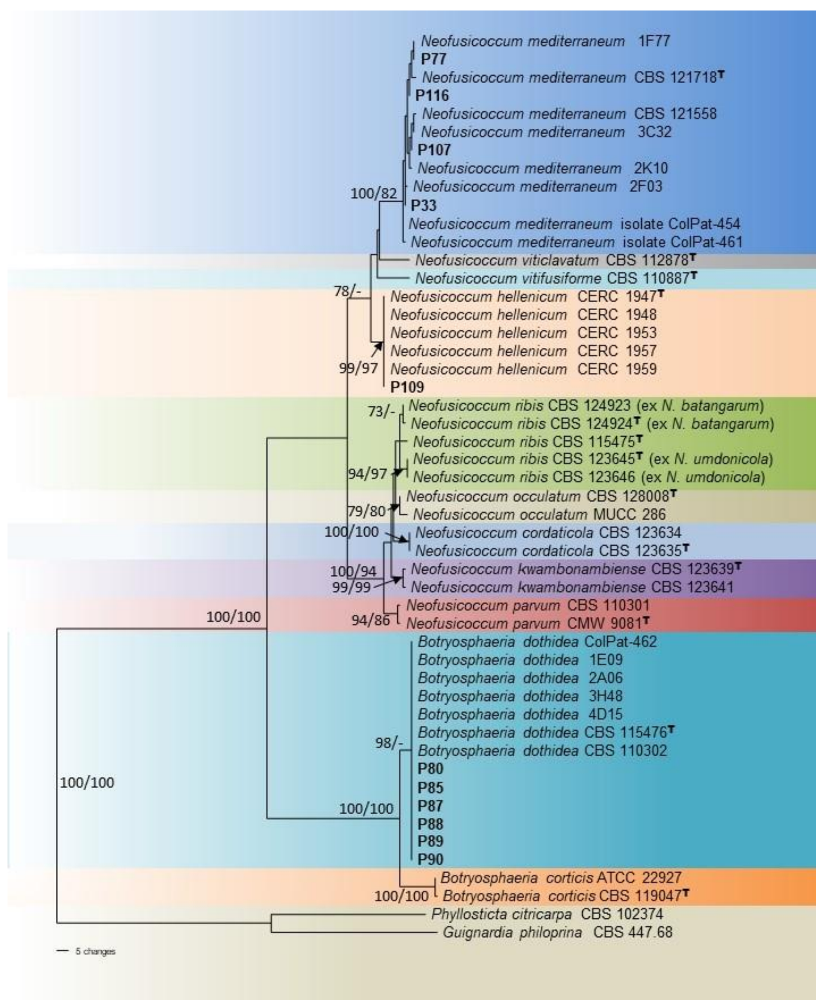

Figure 5. Comparisons in average lesion length (mm) resulting from pathogenicity test among *Botryosphaeria dothidea, Neofusicoccum hellenicum* and *Neofusicoccum mediterraneum* on shoots and fruits of pistachio cv. Bianca. Columns are the means of 12 shoots (three shoots per tree) for each fungal inoculation, and 30 fruit (10 fruit per tree) for each fungal inoculation. 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 (*P* = 0.05).

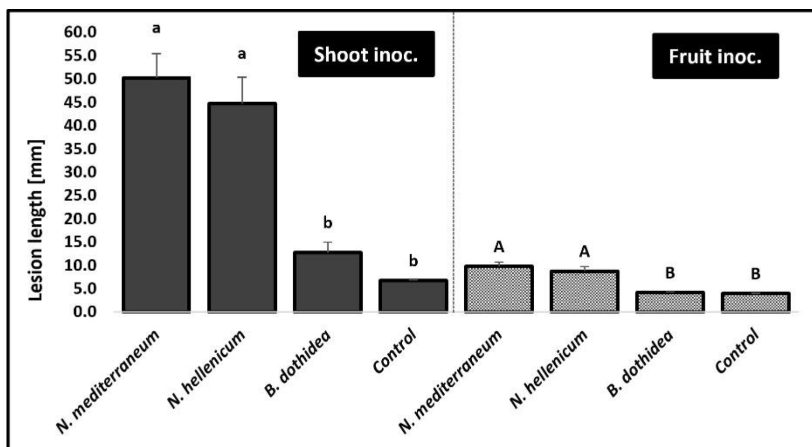

Figure 6. Pathogenicity tests in the field. a, e Control. b, f *Botryosphaeria dothidea*. c, g *Neofusicoccum hellenicum*. d, h *Neofusicoccum mediterraneum*

3.3.3 Phylogenetic analysis

PCR amplification of the ITS region, tef1-α, and tub2 generated 518 to 573, 269 to 290, and 426 to 433 bp fragments, respectively. Alignments of the most prevalent species (60 isolates) revealed three haplotypes for ITS, one for *tef1-α*, and two for *tub2*; therefore, the phylogenetic analyses were performed using a dataset including one isolate representing the haplotypes across the three loci. The ITS, *tef1-α*, and *tub2* sequences generated in this study were deposited in GenBank (Table 1). Results of the partition-homogeneity test indicate no (P = 0.459) significant differences in the three gene datasets. The MP analysis of the combined dataset showed that of 1,439 total characters, 289 were parsimony-informative, 120 parsimony-uninformative, and 1,030 were constant. A total of 47 trees were retained. Tree length was equal to 567, CI = 0.887, RI = 0.970, RC = 0.860. For ML analyses, the best-fit model of nucleotide evolution based on the AIC resulted GTR+I for ITS, HKY+I for *tef1-α* and *tub2*. The ML analysis showed that of 1,439 total characters, 1,030 were constant characters and 333 parsimony informative. Results of both analyses showed that the isolates P80, P85, P87, P88, P89, and P90 grouped with the clade of *B. dothidea* (98/66, MP and ML bootstrap support %, respectively). The isolate P109 grouped with *N. hellenicum* (99/97), and isolates P33, P77, P107, and P116 grouped in the clade of *N. mediterraneum* with high support values (100/82) (Fig. 4).

3.3.4 Pathogenicity test on detached shoots

Preliminary pathogenicity test on detached pistachio shoots revealed that all the three species characterized in this study induced cankers on woody tissues (30 to 35 cm). All the detached shoots were completely infected and necrotized. The species *N. hellenicum* and *N. mediterraneum* were the most aggressive species, inducing severe necrotic lesions of woody tissues. *Botryosphaeria dothidea* induced

lesions all along the entire shoot length, but mostly subcortically. Observation of inoculated shoots showed that *N. hellenicum* produced numerous pycnidia exuding cirri on the surface of the shoots and abundant mass of mycelium. *Neofusicoccum mediterraneum* was also able to produce numerous pycnidia on the shoots, whereas no pycnidia were observed for *B. dothidea*. Controls showed a superficial discoloration of the inoculated area, but no pathogens were isolated. Re-isolations of the inoculated shoots confirmed the identity of the pathogens via morphological observation.

3.3.5 Pathogenicity test in the field

Shoot inoculations conducted in the field confirmed the pathogenicity of the *Botryosphaeriaceae* species of this study. There were significant differences on lesion length on inoculated pistachio shoots between fungal species ($P < 0.05$). Specifically, results showed that mean lesion lengths of *N. hellenicum* and *N. mediterraneum* were significantly different 44.7 and 50.3 mm, respectively, from those in the control (6.8 mm). For both species, the presence of gum leaking from inoculated wounds was observed. Lesion lengths produced by *B. dothidea* (12.8 mm) was not significantly different from that of the control (6.8 mm). Results of the pathogenicity test on fruit confirmed that *N. hellenicum* and *N. mediterraneum* were the most virulent species, inducing lesions of 8.8 and 9.9 mm, respectively, at 5 days post inoculation. *Botryosphaeria dothidea* lesions (4.2 mm) did not differ from control (4.0 mm). Results of pathogenicity tests are shown in Fig. 5 and 6. Re-isolations confirmed the identity of the inoculated pathogens via morphological observation.

3.4 Discussion

Results of our study confirm the presence of three species of *Botryosphaeriaceae* causing disease on pistachio in Italy. Specifically, among five "new" pistachio (i.e., modern plots with 15-30 (or older) years old trees of the female and male cv. Bianca) orchards distributed in Agrigento and Caltanissetta provinces, three species including *B. dothidea*, *N. hellenicum*, and *N. mediterraneum* were characterized.

The species *B. dothidea* was reported affecting pistachio in several countries worldwide (Chen et al. 2014; Inderbitzin et al. 2010; López-Moral et al. 2020b; Mohammadi et al. 2015; Sohrabi et al. 2020; Swart and Botes 1995; Wunderlich et al. 2012). The oldest report of "Botryosphaeria" dieback on pistachio in Italy dates back to 1938, referring to the species *Botryodiplodia pistaciae* (Cristinzio, 1938). In 1972 symptoms of leaf desiccation and panicle lesions were observed in Greece and attributed to the fungus *Camarosporium pistaciae* (Zachos et al. 1974), later transferred to the genus *Botryosphaeria*, and probably identified as *Botryosphaeria ribis* (Chitzanidis,1994). According to Chitzanidis (1994), symptoms observed in Greece were very similar to those caused by *B. dothidea* in California, where the disease was reported for the first time in 1984 (Rice et al. 1985). In this State, numerous studies investigated the biology, the ecology, and the epidemiology of the causal agent, initially identified as *B. dothidea* (Michailides,1991; Michailides and Morgan 1992, 2004). Later, Moral et al. (2010) and Chen et al. (2014) revealed that many fungal species belonging to the *Botryosphaeriaceae* are involved in the Botryosphaeria panicle and shoot blight disease of pistachio in California, of which *N. mediterraneum* is the most widespread. Therefore, *N. mediterraneum* is considered the main causal agent of the disease, but it was previously identified only by morphology as *B. dothidea* (Michailides 1991).

In this study, one of the collected isolates was identified as *N.*

hellenicum. This species was described for the first time in Greece, associated with blighted shoots of pistachio (Chen et al. 2015). This species is considered phylogenetically close to *N. mediterraneum*, *N. viticlavatum*, and *N. vitifusiforme*, which can induce lesion on pistachio branches (Chen et al. 2015). In this study, *N. hellenicum* was occasionally isolated from symptomatic fruit clusters. The results of pathogenicity tests confirm the aggressiveness of this species on woody tissues and fruits. According to our inoculation tests, *N. hellenicum* could be one of the main pathogens of pistachio in the Mediterranean areas, but further studies need to elucidate its geographical and host distribution.

The third species characterized in the present study was *N. mediterraneum*. Isolations results demonstrated that *N. mediterraneum* is widespread in Sicily among pistachio orchards, representing the most frequently recovered species. It was isolated from different symptoms and organs, being able to induce severe lesions on the woody tissues and fruits as well. Species belonging to *Neofusicoccum* were more widespread and aggressive than *B. dothidea* in the shoots inoculations test since the lesions caused by *B. dothidea* did not differ from those of the control, suggesting that the latter species may play a minor role in the disease complex. These results are in accordance with studies conducted in Sicily and Spain on English walnut (Gusella et al. 2021; López-Moral et al. 2020a) and with pathogenicity tests conducted in California (Chen et al. 2014; Moral et al. 2010). The role of *B. dothidea* as a woody pathogen is still uncertain, showing significantly higher virulence in causing lesions on inoculated detached shoots than on trees (Chen et al. 2014; Moral et al. 2010; 2019)). In California, field surveys among mature symptomatic orchards showed that *N. mediterraneum* represent the most frequently encountered species, distributed from northern to southern California (Chen et al. 2014; Moral et al. 2010; Nouri et al. 2019).

Nowadays establishing molecular tools for identifying of causal

agents of fungal diseases represents an essential step forward for fungal taxonomy. It is particularly relevant in terms of canker-pathogens biodiversity and precise epidemiological monitoring, especially during the latent phase of *Botryosphaeriaceae* life cycle. Thus, molecular tools are excellent, not only to detect the pathogen but also to quantify it by using real-time PCR assays (Luo et al. 2017, 2019, 2020).

In addition to species of *Botryosphaeriaceae*, many other fungi have been described worldwide as part of the pistachio canker diseases, such as *Cytospora* spp., *Colletotrichum karstii, Diaporthe* spp., *Didymella glomerata*, *Eutypa* spp., *L. pistaciae*, *Phaeoacremonium* spp., and *Schizophyllum commune* (Aiello et al. 2019; Lawrence et al. 2018; López-Moral et al. 2020b; Mohammadi et al. 2015; Moral et al. 2018; Nouri et al. 2019; Vitale et al. 2018).

The pistachio growing areas investigated in this study fall in the category of "new" plantings if compared to the traditional area of Bronte (Catania Province), where pistachio trees are grafted on wild terebinth in volcanic soil (natural plantings). *Botryosphaeriaceae* spp. have been sporadically detected in Bronte (data not shown), but their presence has not to date represented a risk for pistachio growers. *Botryosphaeriaceae* with broad host ranges, do not infect the same host in all places where they co-occur, probably due to local environmental factors, niche competition with other endophytes, physiological or physical variation in the host of different regions (Moral et al. 2019; Slippers and Wingfield, 2007). In this regard, for instance, leaf wetness data (monthly minutes average) collected from March to August of 2019 by the weather stations of SIAS (Servizio Informativo Agrometeorologico Siciliano) located in the Agrigento Province (new plantings) and the commune of Bronte (Catania Province) (traditional plantings) showed a relevant difference Specifically, values of the leaf wetness duration were more than triple in Agrigento than in Bronte.

Studying the environmental factors involved in the disease

cycle by the *Botryosphaeriaceae* members is crucial to predict the disease pressure and properly manage fungicide sprays in the field (Morgan et al. 2009). In California, for instance, the forecast based on leaf wetness (LWM) is very popular among the pistachio growers (Morgan et al. 2009), and walnut growers have started using the LWM as well (Michailides et al. 2015). In Agrigento, *Botryosphaeriaceae* induced severe symptoms and a high level of disease in the "new" farms surveyed in this study. For instance, one of the orchards of this study suffered a production loss up to 50% and a severe defoliation 30 to 50 days before the harvest.

Orchards investigated in this study were often near wild hosts (cedar, eucalyptus, willows, etc.), that represent a source of inoculum of *Botryosphaeriaceae* (Inderbitzin et al. 2010; Ma et al. 2001). In recent years, *Botryosphaeriaceae* spp. have been detected in Sicily in many different crops, some of which are considered emerging, like the sub-tropical crops, i.e. avocado and mango (Guarnaccia et al. 2016; Ismail et al. 2013). The presence of *Botryosphaeriaceae* infecting one crop represents important epidemiological information since these species are highly polyphagous and able to infect multiple hosts. This fact occurs, especially in Mediterranean Countries where many essential crops are cultivated contiguously, allowing a flow of inoculum among the hosts (Moral et al. 2019).

Until now, our observations conducted in Sicily revealed, that in the traditional area of Bronte, rather than in the new plantings, *L. pistaciae* was frequently isolated from symptomatic woody tissue, whereas *Botryosphaeriaceae* spp. were rarely detected. Otherwise, in the new plantings, *Botryosphaeriaceae* were frequently isolated, possibly representing the major concern for the growers. Further investigation will be necessary to elucidate the distribution, the diversity and control of *Botryosphaeriaceae* species. This study represents the first updated investigation on the etiology of *Botryosphaeriaceae* disease on pistachio in Italy, and the first report of the species *N. hellenicum* in Italy.

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4. Characterization of *Eutypa lata* and *Cytospora pistaciae* causing dieback and canker of pistachio in Italy

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4.1 Introduction

Pistachio is cultivated in the southern regions of Italy, of which Sicily is the main production area. The province of Catania (with 430 ha of pistachio), followed by the provinces of Caltanissetta (with 220 ha) and Agrigento (with 145 ha) are the largest pistachio-producing areas, with a total production of 3,878 tons (AGRISTAT, 2017). Currently, the commune of Bronte in Catania Province represents the most important area of Sicily for pistachio production, and pistachio is an important economic resource for this territory (Barone and Marra, 2004). In this area, different pistachio cultivars are grafted on terebinth plants which are grown on volcanic soils (Barone *et al.*,

1985). Few studies have been conducted to investigate pistachio diseases occurring in Italy, and only a few diseases have been reported to date. These include branch dieback (caused by *Botryodiplodia* sp.), leaf spot (*Alternaria alternata*), anthracnose, branch and twig cankers (*Botryosphaeria dothidea*) and phylloptosis and leaf spots (mainly caused by *Septoria pistaciae*) (Casalicchio, 1963; Schilirò and Privitera, 1988; Frisullo *et al*., 1996; Vitale *et al*., 2007). In east- ern Sicily, cankers and decline caused by *Liberomyces pistaciae* Voglmayr, Vitale, Aiello, Guarnaccia, Luongo & Belisario are the most important pistachio diseases (Vitale *et al*., 2018). Blight caused by *Arthrinium xenocordella* Crous was also recently reported on pistachio fruit in the Agrigento Province (Aiello *et al*., 2018).

During the winter of 2017, pistachio trees with die- back, canker and gummosis symptoms were observed in the area of Bronte. Following culturing from necrotic tissues, two fungal species were consistently isolated. Cankers from one orchard generated colonies of *Cytospora* while cankers from a second orchard generated *Eutypa* colonies.

The aim of the present study was to investigate the etiology of pistachio canker diseases, which could represent new threats for the pistachio production of Sicily.

4.2 Materials and Methods

4.2.1 Isolation and morphology of fungi

Surveys were conducted in ten pistachio orchards with histories of branch canker and dieback in east- ern Sicily (Catania Province). Approximately 20 symptomatic pistachio branches with canker were collected from each orchard for analyses. Sub-cortical and wood fragments (about 5 × 5 mm) were cut from the margins between

affected and healthy branch tis- sues. Tissue pieces were disinfected in 1.2% sodium hypochlorite for 60 s, rinsed in sterile water and dried on sterile filter paper. The fragments were then placed into Petri plates containing potato dextrose agar (PDA, Oxoid) amended with 100 mg L-1 of streptomycin sulfate (Sigma-Aldrich), and incubated at room temperature (25 ± 5°C). Fungal colonies consistently growing from symptomatic tissues were cultured into new PDA plates. To obtain pure cultures, single- conidium or hyphal-tip isolations were performed after 1 month incubation at room temperature under natural light conditions. Isolates for each putative fungal pathogen (four isolates of *Eutypa* and three of *Cytospora*) were characterized by morphological, molecular and phylogenetic analyses (Table 1). These cultures were deposited in the working collection of Dr Pedro Crous (CPC), at the Westerdijk Fungal Bio- diversity Institute, Utrecht, The Netherlands (Table 1). Size and shape of conidia were recorded for each fun- gal isolate grown on PDA for 2 weeks at 25 ± 1°C.

4.2.2 DNA extraction, PCR amplification and sequencing

Extractions of genomic DNA were performed from pure cultures, as reported elsewhere (Guarnaccia and Crous, 2017), using the Wizard Genomic DNA Purification Kit (Promega Corporation). Partial regions of five loci were amplified. The primers ITS5 and ITS4 (White *et al*., 1990) were used to amplify the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA operon, including the 3' end of the 18S rRNA, the first internal transcribed spacer region, the 5.8S rRNA gene; the second internal transcribed spacer region and the 5' end of the 28S rRNA gene. The primers ACT- 512F and ACT-783R (Carbone and Kohn, 1999) were used to amplify part of the actin gene (*act*). The partial beta-tubulin (*tub2*) gene was amplified with primers Bt- 2a and Bt-2b (Glass and Donaldson, 1995). The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used to amplify part of the translation elongation factor 1-α gene (*tef1*). The primers

5f2/7cr were used to amplify part of *rpb2* (O'Donnell *et al*., 2010). The regions ITS, *act*, *tef1* and *rpb2* were amplified for the species of *Cytospora* using the PCR programmes adopted by Lawrence *et al*. (2018) and Jami *et al*. (2018). The regions ITS and *tub2* were amplified for the species of *Eutypa* following the PCR programmes used by Moyo *et al*. (2018a). The PCR products were sequenced in both directions using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems Life Technologies), after which amplicons were purified through Sephadex G-50 Fine columns (GE Healthcare) in MultiScreen HV plates (Millipore). Purified sequence reactions were analyzed on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies). The DNA sequences generated were analyzed and consensus sequences were computed using the program SeqMan Pro (DNASTAR).

4.2.3 Phylogenetic analyses

Novel sequences generated in this study were blast- ed against the NCBIs GenBank nucleotide database, to determine the closest relatives to be included in the phylogenetic analyses. Blast analyses indicated that three isolates belonged to *Cytospora* and the remaining four to *Eutypa*. Sequence alignments of the different gene regions, including sequences obtained from this study and sequences from GenBank, were initially performed using the MAFFT v. 7 online server (http://mafft.cbrc. jp/alignment/server/index. html) (Katoh and Stand- ley, 2013), and then manually adjusted in MEGA v. 7 (Kumar *et al*., 2016). To establish the identity of the fun- gal isolates, phylogenetic analyses were conducted using one locus (data not shown) as well as concatenated analyses of four loci (ITS, *act*, *tef1* and *rpb2*) for *Cytospora* spp. and two loci (ITS and *tub2*) for *Eutypa* spp., as indicated by blast analysis. Additional reference sequences were selected based on recent studies on *Cytospora* and *Eutypa* species (Lawrence *et al*., 2018, Moyo *et al*., 2018a, b). Phylogenetic

analyses were based on Maximum Parsimony (MP) for all the individual loci and for the multi-locus analyses. The MP analyses were carried out using PAUP (Swofford, 2003). Phylogenetic relationships were estimated by heuristic searches with 100 random addition sequences. Tree bisection-reconnection was used, with the branch swapping option set on 'best trees' only, with all characters weighted equally and alignment gaps treated as fifth state. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC) were calculated for parsimony and the boot- strap analyses (Hillis and Bull 1993) were based on 1,000 replicates. Sequences generated in this study were deposited in GenBank (Table 1, see the publication Doi: 10.13128/Phyto-10880).

4.2.4 Pathogenicity of representative isolates

Pathogenicity tests with one representative isolate of *C. pistaciae* (CPC34208) and one of *E. lata* (CPC34213; Table 1) were carried out to satisfy Koch's postulates. These tests were carried out in a growth chamber maintained at 25 ± 1°C. Potted 5-y-old plants of *P. vera* grafted onto *P. terebinthus* were used for artificial inoculations. Three plants were inoculated with each iso- late. Six wounds were made on individual plant stems approx. 8-10 cm apart from each other. Inoculations were made on stems after removing of bark discs with a cork borer, placing a 5 mm plug from a 14-d-old PDA culture of test isolate into the wound and covering with Parafilm® (Pechney Plastic Packaging Inc.) to prevent desiccation. An equivalent number of plants and inoculation sites were inoculated with sterile PDA plugs to serve as controls. The inoculated plants were observed once each month for symptoms development, and a final assessment was conducted 5 months after inoculation. To fulfil Koch's postulates, re-isolations were carried out following the procedure described above, where tissue fragments were plated onto PDA. Each re-isolated fungus

was identified through morpho- logical characteristics.

4.3 Results and Discussion

Symptomatic plants showed cankers with cracking and gum exudation, and often branches or shoots showed dieback. Under the bark of affected branches, cankers were characterized by discolouration and necrosis, and in some cases discolouration extended to the vascular tissue (xylem) and pith. Two different fun- gal colony types were consistently obtained from isola- tions from symptomatic tissues (Figure 1) taken from the two orchards. Cankers from one orchard generated *Cytospora* colonies while cankers from the other orchard generated *Eutypa* colonies. The same symptoms in the remaining orchards investigated in the Bronte area pro- duced colonies of *L. pistaciae* (Vitale *et al*., 2018).

Conidia of three representative isolates of *Cytospora* were in accordance with the description by Lawrence *et al*. (2018) of *C. pistaciae* Lawr., Holland & Trouillas. The four MP trees derived from the single gene sequence alignments (ITS, *act*, *tef1* and *rpb2*) were topologically similar, confirming that the three isolates used for the molecular analyses were *Cytospora*. The combined phyogeny of *Cytospora* species consisted of 35 sequences, including the outgroup sequences of *Diaporthe limonicola* (culture CBS 142549; Guarnaccia and Crous, 2017). A total of 2,056 characters (ITS: 1–574, *act*: 581–890, *tef1*: 897–1289, *rpb2*: 1296–2056) were included in the phylogenetic analysis of *Cytospora* spp. For the phylogeny of *Cytospora* species, 489 characters were parsimony-informative, 336 were variable and parsimony uninformative and 1,213 characters were constant. A maxi- mum of 1,000 equally most parsimonious trees were saved (Tree length = 1 552, CI = 0.743, RI = 0.782 and RC = 0.581). Bootstrap support values from the parsimony analysis were plotted on the phylogenetic trees presented in Figure 2. In the combined analyses, the three representative isolates clustered with four reference strains

of *C. pistaciae*. The individual alignments and trees of the single loci used in the analyses were compared with respect to their performance in species recognition. *Cytospora pistaciae* was differentiated and identified in all single-gene analyses.

Cytospora terebinthi Bres. has been reported in Italy as the causal agent of cankers and gummosis of pistachio (Corazza *et al*., 1990; Furnitto, 1984), while other *Cytospora* species have been reported in other crops, including peach (Hampson and Sinclair., 1973; Banko and Helton, 1974). The taxonomy of *Cytospora* species associated with fruit and nut crops was recently revised, and *C. pistaciae* was described as a new species on pistachio in California, but the pathogenicity of this species was not investigated (Lawrence *et al*. 2018).

Conidia of four isolates of *Eutypa* were in accordance with the description of *E. lata* by Moyo *et al*. (2018b). The two MP trees derived from the single gene sequence alignments (ITS and *tub2*) were topologically similar, and this confirmed that the four isolates used in this study were *Eutypa*. All the species belonging to *Eutypa* and other Xylariales used in the multi- locus phylogeny consisted of 29 sequences with the outgroup sequences of *L. pistaciae* (CBS 144255; Vitale *et al*., 2018). A total of 1,076 characters (ITS: 1–582, *tub2*: 589–1,076) were used for the Xylariales analysis, and 453 characters were parsimony-informative, 166 were variable and parsimony-uninformative and 451 characters were constant. A maximum of 1,000 equally most parsimonious trees were saved (Tree length = 1 669, CI = 0.648, RI = 0.786 and RC = 0.509). Bootstrap support values from the parsimony analysis were plotted on the phylogenetic trees presented in Figure 3. In the combined analyses, the four isolates were related to reference isolates of *E. lata*. The individual alignments and trees of the single loci used in the analyses were com- pared with respect to their performance in species recognition. *Eutypa lata* was differentiated and identified in all single-gene analyses.

Eutypa lata is a pathogen with a wide host range, occurring in more than 160 hosts (Farr and Rossman, 2017). In Italy, *E. lata* has been reported on *Acer* sp. in Sicily (Greuter *et al*., 1991), *Ribes rubrum* (Prodorutti *et al*., 2008), olive trees (Tosi and Natalini, 2009) and *Vitis vinifera* (Acero *et al*., 2004). Eutypa dieback and gummosis of pistachio caused by *E. lata* has been reported only in Greece (Rumbos, 1986).

Five months after artificial inoculation, symptoms produced from each fungus in trees were similar to those present on trees in the field. These consisted of external cankers and gummosis produced around the inoculation sites, with small cracks present in each sunken lesion. After removing the bark, a dark discolouration and necrotic tissues were visible (Figure 1). The respective inoculated pathogens were re-isolated from symptomatic tissues, thus fulfilling Koch's postulates. No symptoms were observed on control (uninoculated) plants. This is the first report of *E. lata* and *C. pistaciae* associated with cankers on pistachio in Europe. Further studies should investigate the role of propagation mate- rial, mechanical injuries and pruning wounds in disease transmission and spread.

Figure 1. Symptoms reproduced from mycelial plug inoculation with *Cytospora pistaciae* (a) and *Eutypa lata* (b) on 5-y-old potted plants of *Pistacia vera* 5 months after inoculation with respective fungi. Cultural characteristics of *Cytospora pistaciae* (c) and *Eutypa lata* (d) colonies grown on PDA are also illustrated.

Figure 2. The first of two equally most parsimonious trees obtained from a heuristic search of the combined ITS, act, *tef1* and *rpb2* sequence alignments of *Cytospora* spp. Bootstrap support values are shown at the nodes. The strains isolated in this study are show are shown in red and the scale bar represents the number of changes. The tree was rooted to *Diaporthe limonicola* (CBS 142549).

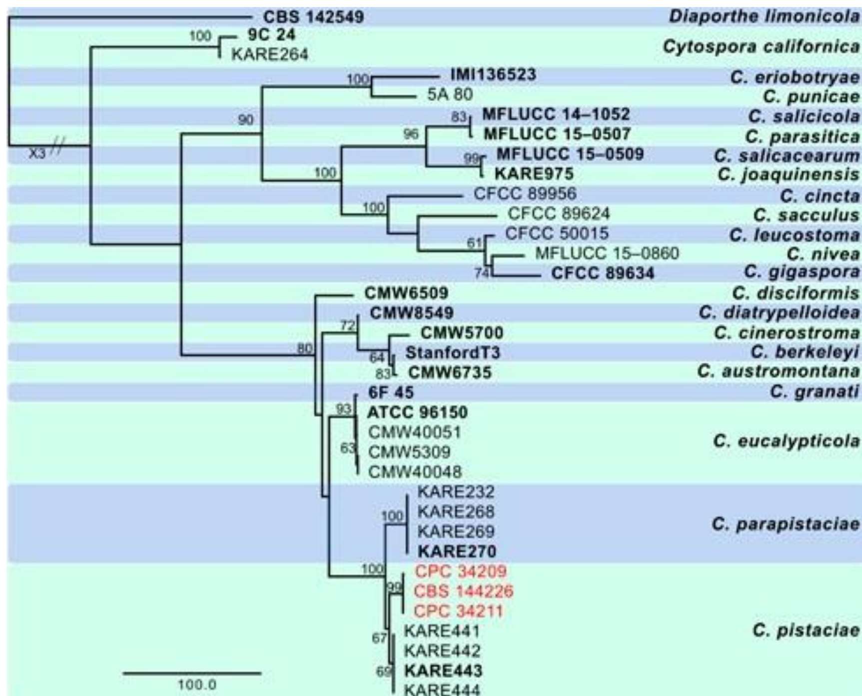
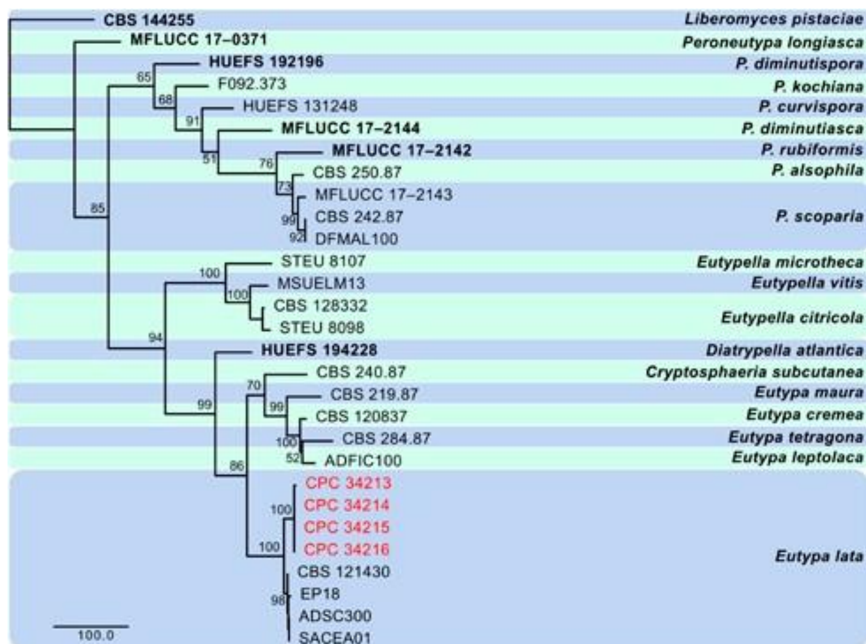


Figure 3. The first of four equally most parsimonious trees obtained from a heuristic search of the combined ITS, and *tub2* sequence alignments of species belonging to *Eutypa* and other genera of *Diatrypaceae*. Bootstrap support values are shown at the nodes. The strains isolated in this study are shown in red and the scale bar rep- resents the number of changes. The tree was rooted to *Liberomyces pistaciae* (CBS 144255).



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5. Other research activities: First reports

5.1 First Report of Fruit Blight Caused by Arthrinium xenocordella on Pistacia vera in Italy

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Pistacia vera L. is a nut tree native to Central Asia and the Middle East widely cultivated in Sicily, Italy. In July 2017, a new disease was observed on approximately 450, 70-year-old *P. vera* plants (var. "Bianca"), in a commercial orchard in Western Sicily (Agrigento Province), Italy. More than 30% of the fruits showed disease symptoms. The initial symptom was external brown discoloration on the epicarp of immature fruits. As the disease progressed, the discoloration turned dark-brown to black and covered up to 50% of the fruit surface. As a consequence, infected fruit was blighted and cling onto the shoot. Internal fruit tissues showed discoloration of the endocarp, sometimes leading to darkening of the kernel. Small sections of thirty diseased fruits were surface disinfected for 1 min in 1.5% sodium hypochlorite solution, rinsed in sterile water, placed on potato dextrose agar (3.9% PDA, Oxoid) amended with 100 mg/liter of streptomycin sulfate (Sigma-Aldrich), and then incubated at 25 ± 1°C for seven days. A fungus was consistently isolated from

affected tissues of fruits forming flat colonies, with moderate aerial mycelium and surface pale luteous with patches of olivaceous-grey, reverse pale luteous. Single-spore isolates on PDA produced conidia with a globular to ellipsoid shape, (7–) 9–10 (–11) µm long and 6–7 µm wide. Setae were erect, brown, subcylindrical, 1- septate, with a truncate base, 100 µm tall, 5–8 µm in diam, straight to irregularly curved. The identification of 4 representative isolates was determined by partial sequencing of the rDNA internal transcribed spacer (ITS) region and β-tubulin (TUB) gene, as described in Crous and Groenewald (2013). The obtained ITS sequence (GenBank Accession No. MG921593) and *tub2* sequence (GenBank Accession No. MG921594) of the isolate CPC33875 (Di3A-PV72) showed 100% and 99% identity with those of *Arthrinium xenocordella* tester isolate (KF144925 and KF145013), respectively. Pathogenicity tests were conducted on immature fruits of *P. vera*. Thirty fruits were inoculated by spraying conidial suspension (1 × 105 conidia/ml) of one isolate (Di3A-PV72) obtained from a 15- day-old culture. The same number of fruits were sprayed with sterile distilled water and served as controls. Plants were kept at 25 ± 1°C and 95% relative humidity on a 12-h fluorescent light/dark regimen. After seven days, the tested isolate caused symptom identical to those observed in the field. Moreover, approximately 50% infected fruits were covered by whitish mycelium of the fungus. Control fruit were asymptomatic. *A. xenocordella* was re-isolated from the infected fruit and identified as previously described. *A. xenocordella* was recently described from soil in Zimbabwe (Crous and Groenewald 2013) and *Arthrinium* spp. have been reported as pathogens on kernel of barley and on wheat (Martínez-Cano et al. 1992; Mavragani et al. 2007). To our knowledge, this is the first report of *A. xenocordella* as plant pathogen and causing fruit blight on *P. vera.*

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5.2 ***First report of leaf and twig blight of Indian hawthorn (Rhaphiolepis indica) caused by Neofusicoccum parvum in Italy***

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During the winter of 2018 symptoms of leaf and twig blight were observed on about 15% of 7500 4-year-old potted plants of Indian hawthorn [*Rhaphiolepis indica* (L.) Lindl.] grown in a commercial nursery in Catania province, Italy. Necrosis was found in the petioles, moving upward through the mid rib and blade, showing brownish V-shape lesion at the bottom of the leaves and leading to a complete blight of the twigs. Under the bark, discolored tissue was found. Diseased tissues were surface disinfected for 1 min in 1.5% sodium hypochlorite solution, rinsed in sterile water, placed on PDA amended with 100 mg/l of streptomycin sulfate, and then incubated at 25 °C for seven days. A fungus like *Neofusicoccum* sp. was consistently isolated. ITS region of rDNA and part of *tef1* gene were amplified using ITS5, ITS4 and EF1-728F, EF1-986R primers, respectively. The sequence data were deposited in GenBank. BLAST analysis identified the fungus as *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips. The obtained ITS sequences (GenBank accession Nos. MN128645; MN128646) of the isolates CBS 145794, CBS 145795 showed 99.65% and 99.83% identity with the tester isolate (KJ657701) respectively, and *tef* sequences (MN175951; MN175952) showed 100% and 98.97% (KJ126847). The isolates have been registered in the CBS Westerdijk

Fungal Biodiversity Institute collection (Utrecht). Pathogenicity tests were conducted inoculating 10 twigs with a mycelial plug. Control consisted of sterile PDA plugs. Plants were moved into a growth chamber at 25 °C ± 1 °C. The same symptoms observed in field appeared on 90% of the inoculated twigs after 10 days and complete twigs blight of the apical part of the plant after 17 days (Fig.1). Re-isolations completed Koch's postulates. This fungus is spreading in Sicily on different important crops (Guarnaccia et al. 2016; Ismail et al. 2013) but for our knowledge, this is the first report of leaf and twig blight caused by *N. parvum* on Indian hawthorn.

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Figure 1. Results of pathogenicity test.

6. Other research activities on *Botryosphaeriaceae* diseases: *Botryosphaeriaceae* species causing canker and dieback of English walnut (*Juglans regia*) in Italy

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6.1 Introduction

English walnut (*Juglans regia* L.) is an important tree crop, cultivated for nuts and for wood production in many regions of the world. Nowadays, production of the nut crop is increasing, with annual yields of over 880,800 metric tons. China and the United States are the biggest producers, at 40% and 31%, respectively, of the global total (INC-Statistical Yearbook, 2018). Italy is considered a suitable country for production of a range of nuts, including almond, chestnut and hazelnut, but since the 1970s, walnut production declined consider- ably, and Italy changed from being a prominent producer to become one of the top walnut importers globally (Calcagni, 2019). Although the history of walnut production in Italy cannot be described as successful, recently production was revived, with new plantings in

Centre-Northern Italy, characterized by specialized orchards, cutting-edge agronomic techniques, and mechanical harvesting (Calcagni, 2019). Common fungal pathogens affecting this crop are the ascomycete *Ophiognomonia leptostyla* (Syn. *Marssonina juglan- dis*) cause of walnut anthracnose, and *Geosmithia morbida*, cause of 'thousand cankers disease' (TCD), a serious pathogen vectored by the walnut twig beetle *Pityophthorus juglandis*. In the recent past, much research in walnut-producing regions globally has focused on canker and shoot blight caused by species of *Botryosphaeriaceae*. In California, isolations from stem and shoot cankers, shoot, twig, leaf, and fruit blight, necrotic leaf lesions, and black cankers around the graft union included 10 species of *Botryosphaeriaceae*, including *Botryosphaeria dothidea*, *Diplodia mutila*, *Diplodia seriata*, *Dothiorella iberica*, *Lasiodiplodia citricola*, *Neofusicoccum mediterraneum*, *Neofusicoccum nonquaesitum*, *Neofusicoccum parvum*, *Neofusicoccum vitifusiforme* and *Neoscytalidium dimidiatum* (Chen *et al.,* 2013, Chen, Morgan, Hasey *et al.,* 2014; Inderbitzin *et al.,* 2010; Michailides & Hasey, 2010; Trouillas *et al.,* 2010). In China and Korea, investigations on stem and branch canker, dieback, kernel decay, canker on seed- lings and fruit rot showed the presence of *B. dothidea*, *Lasiodiplodia pseudotheobromae* and *N. parvum* (Chen *et al.,* 2019; Cheon *et al.,* 2013; Li *et al.,* 2016; Yu *et al.,* 2015). Recently, also on the European Continent, in Spain and in the Czech Republic, symptoms of cankers, branch dieback and shoot blight were associated with several fungal species including *B. dothidea*, *D. seriata*, *Dot. omnivora*, *Dot. sarmentorum*, *N. mediterraneum* and *N. parvum* (Eichmeier *et al.,* 2020; López-Moral *et al.,* 2019; Moral *et al.,* 2010). Further reports of 'Botryosphaeria blight' came from other countries, including Iran, Egypt and Greece (Abdollahzadeh *et al.,* 2013; Haggag *et al.,* 2007; Rumbos, 1987).

In Sicily (southern Italy), many growers during the 1990s were encouraged to invest in walnut timber production, using funding from the EU Common Agricultural Policy, but often the desired results

were not achieved for a variety of reasons based on environmental conditions and good agronomic management (La Mantia *et al.,* 2008). Although Belisario (1996) reviewed the main dis- eases affecting walnut in Italy, there are several outdated reports of Botryosphaeria canker diseases. Some reports of walnut canker and dieback were attributed to *Diplodia juglandis*, *Phomopsis* spp., *Melanconis juglandium*, *Phoma juglandina* and *Cytospora juglandina* (Belisario, 1996; Ciccarone, 1987; Sisto & Luisi, 1983; Vercesi, 1982). For example, in 1994 canker and dieback of walnut branches and twigs were reported in Apulia (southern Italy) and attributed to *Botryosphaeria ribis* (Frisullo *et al.,* 1994), but molecular data were not provided to confirm the identity of the pathogens.

A survey conducted in a 30-year-old walnut orchard in Sicily in October of 2019, revealed the presence of symptoms including cankers, exudates, wood discoloration and dieback. The aim of this study was to identify fungi associated with these symptoms and test their pathogenicity, in order to better understand the syndrome ae-tiology, which could represent a serious threat for an important and expanding crop like walnut in Italy.

6.2 Materials and Methods

6.2.1 Field survey and isolations

In October 2019, a survey was carried out in a 30-year-old walnut orchard planted for wood production in the Sicilian hinterland (San Giuseppe Jato-Palermo Province). The walnut orchard, planted on hilly land of 5 hectares in area, included 1,000 trees of which up to 30% showed the presence of cankers and dieback. Samples of symptomatic branches or pieces of trunk were collected from seven randomly selected trees and transferred to the laboratory for analy-ses. Small sections of symptomatic phloem and xylem from the mar-

gin of diseased and healthy tissues were surface sterilized for 1 min in 1.5% sodium hypochlorite solution, rinsed in sterile water, placed on potato dextrose agar (PDA, Lickson) amended with 100 mg/L of streptomycin sulphate (Sigma-Aldrich) to prevent bacterial growth and incubated at 25 ± 1°C for 4 days. Direct isolations from asexual structures (pycnidia) found on bark tissues were also performed and compared with results of isolations from symptomatic tissues. Single hyphal tip isolates were obtained from pure cultures grown on PDA at 25°C.

6.2.2 Morphological characterization and effect of temperature on mycelial growth rate

Three representative isolates of each morphologically different group were selected and used for further morphological and molecular characterization, and for pathogenicity tests. These isolates, according to their group, were named: WT1-1 (*N. parvum*), WT4-1 (*N. mediterraneum*) and WT7-5 (*B. dothidea*). To study conidial morphology, sporulation was induced by transferring single hyphal isolates onto Technical Agar (AT, 1.2% Agar, Oxoid) with sterilized pine needles or pistachio leaves placed on the surface, and incubating at room temperature. After 2 weeks, pycnidia were mounted in water and 50 conidia were measured using a light microscope (Axioskop; Zeiss) coupled to an AxioCam MRc5 (Zeiss) digital camera; images were captured using Axio-Vision 4.6 (Zeiss) software. Conidial dimensions are presented here as 5th and 95th percentiles with extreme values in parentheses, L/W ratios, means and standard deviations (*SD*). To determine the effect of temperature on mycelial growth, agar plugs (6-mm-diameter) were cut from the margin of 7-day-old colonies of the same isolates chosen for the analysis. Plugs of each isolate were transferred to PDA in Petri dishes and incubated at 5, 10, 15, 20, 25, 30, 35 and 40°C in the dark. Three replicates for each isolate and temperature combination were pre- pared, and the

experiment was repeated once. Colony diameter was measured along two perpendicular axes when the colony reached at least two-thirds of the plate diameter, and the data were converted to daily radial growth in mm/day. After eleven days, cultures incubated at temperatures in which there was no growth were placed at 25°C to determine whether these temperatures were fungistatic or fungicidal. For each isolate, average data for radial growth were adjusted to a regression curve using Statgraphics Plus 5.1 software (Manugistics Inc.) and the best polynomial model was chosen based on parameter significance ($p < .05$) and coefficient of determination (R^2) to estimate the optimum growth temperature of each isolate.

6.2.3 Molecular characterization and phylogenetic analysis

Genomic DNA of the selected isolates was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation). The internal transcriber spacer region (ITS) of the nuclear ribosomal RNA cluster was amplified with primers ITS5 and ITS4 (White *et al.,* 1990). Primers EF1-728F and EF1-986R (Carbone & Kohn, 1999) were used to amplify part of the translation elongation factor 1α gene (*tef1*-α), while the primer set Bt-2a and Bt-2b (Glass & Donaldson, 1995) was used for the partial β-tubulin gene (*tub2*). PCR amplification conditions were set as follows: initial denatur- ation temperature of 94°C for 5 min, followed by 35 cycles at the de- naturation temperature of 94°C for 30 s, annealing temperature of 48°C for ITS and 52°C for *tef1*-α and *tub2* for 50 s, extension at 72°C for 2 min, and final extension at 72°C for 7 min. PCR products were purified and sequenced in both directions by Macrogen Inc. (South Korea). Sequences were edited using Sequencher software (version 4.7, Gene Codes Corporation). Before constructing the phylogenetic tree, BLAST searches were performed using the NCBI nucleotide database (Altschul *et al.,* 1997). ITS, *tef1*-α and *tub2* DNA sequence datasets were aligned using ClustalX v. 1.83 (Thompson *et al.,* 1997), and

MEGAX: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar *et al.,* 2018), and manual adjustments of alignments made when necessary. A partition-homogeneity test with heuristic search and 1,000 homogeneity replicates was per- formed using PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0a to test for discrepancies among the three genes dataset. For comparison, 34 additional sequences were selected according to the taxonomic classification study on the *Botryosphaeriaceae* genera and species provided by Phillips *et al.,* (2013), and previous work on *Botryosphaeriaceae* affecting walnut (Chen, Morgan, Hasey *et al.,* 2014; López-Moral *et al.,* 2019) to be included in the alignment (Table 1). Maximum parsimony (MP) analysis was performed in PAUP v.4.0a. The analysis of the combined dataset (ITS+ *tef1*-α + *tub2*) 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 consistence index (RC) were calculated. A total of 1,000 bootstrap replicates were performed to test the robustness of the tree topologies. MrModeltest v. 2.4 (Nylander, 2004) was used to identify the best-fit model of nucleotide evolution for each gene according to the Akaike information criterion (AIC). The maximum likelihood analysis (ML) of the combined genes was performed in GARLI v.0.951 (Zwickl, 2006), and clade support was assessed by 1,000 bootstrap replicates. *Guignardia philoprina* (CBS 447.68) and *Phyllosticta citricarpa* (CBS 102374) served as outgroups in both analyses.

6.2.4 Pathogenicity test on detached fruits

In order to fulfil Koch's postulates, three replicates of two green fruits for each fungal isolate (WT1-1, WT4-1, WT7-5) were used for

pathogenicity tests. Three green fruits, approximately 4 × 6 cm in diameter, were used for controls. Fruits were surface disinfested in 1.2% sodium hypochlorite solution for 1 min and rinsed in sterile water. Once completely air-dried under a laminar hood, a piece of husk was removed with a 6-mm-diameter cork borer and 6-mm-diameter mycelial plugs taken from a 14-day-old cultures were applied to the wound on each fruit at a depth of 2–3 mm. Wounds were covered with Parafilm® (Pechney Plastic Packaging Inc.) to prevent desiccation. Controls consisted of sterile PDA plugs applied to wounds. All replicates were kept in plastic crisper to maintain high humidity in a growth chamber with a 12 hr photoperiod at 25°C ± 1°C. The presence and diameter of the resulting lesions was recorded 7 days after inoculation.

6.2.5 Pathogenicity test on seedlings

Pathogenicity tests were conducted on twelve potted, healthy, 1-year-old seedlings of walnut (*J. regia*). Three replicates were used for each fungal isolate tested. Three plants were used for controls. A piece of bark was removed with a 6-mm-diameter cork borer, and 6-mm-diameter mycelial plugs taken from a 13-day-old culture of the same isolates used for pathogenicity test on fruits were applied at a depth of 2–3 mm to two separate wounds on each seedling, one inoculation point at 9–10 cm from the top of the plant, and one at 7–8 cm above the root collar. Wounds were covered with Parafilm® (Pechney Plastic Packaging Inc.) to prevent desiccation. Controls consisted of sterile PDA plugs applied to wounds. All replicates were maintained in a growth chamber with a 12 hr photoperiod at 25°C ± 1°C and watered regularly. After 1 month, the lengths of necrotic lesions upwards and downwards from the point of inoculation were measured after scraping away the bark cortex and exposing the internal lesions on the vascular cambium. To fulfil Koch's postulates, attempts were made to re-isolate the inoculated fungi by transferring

pieces of wood taken from the mar- gins of each lesion to PDA. Cultures were incubated at 25°C until fungal colonies developed. The re-isolated fungi were identified from their micro-morphological characters.

Table 1. GenBank accession numbers of the species used in the phylogenetic analysis

		GenBank accessions		
Species	**Strain**	**ITS**	***tef1-α***	***tub2***
Botryosphaeria dothidea	CMW8000	AY236949	AY236898	AY236927
	CBS110302	AY259092	AY573218	EU673106
	2E55	KF778783	KF778973	KF778878
	2K23	KF778784	KF778974	KF778879
	6I19	KF778786	KF778976	KF778881
	5 A02	KF778787	KF778977	KF778882
	ColPat446	MK431836	MK461890	MK522083
	ColPat530	MK431843	MK461897	MK522084
	ColPat553	MK431844	MK461898	MK522085
	WT7-5	MT252674	MT265238	MT265235
Botryosphaeria corticis	CBS119047	DQ299245	EU017539	EU673107
	ATCC 22927	DQ299247	EU673291	EU673108
Guignardia philoprina	CBS 447.68	AY236956	AY236905	AY236934
Neofusicoccum batangarum	CBS 124924	FJ900607	FJ900653	FJ900634
	CBS 124923	FJ900608	FJ900654	FJ900635
Neofusicoccum cordaticola	CBS 123634	MH863316	EU821868	EU821838
	CBS 123635	EU821903	EU821873	EU821843
Neofusicoccum kwambonambiense	CBS 123639	MH863317	EU821870	EU821840
	CBS 123641	EU821919	EU821889	EU821859
Neofusicoccum mediterraneum	CBS_121718	GU251176	GU251308	GU251836
	CBS 121558	GU799463	GU799462	GU799461
	1L85	KF778818	KF779008	KF778913
	2E54	KF778836	KF779026	KF778931
	6I27	KF778848	KF779038	KF778943
	WT4-1	MT252673	MT265237	MT265234
Neofusicoccum occulatum	CBS 128008	MH864743	EU339509	EU339472
	MUCC286	EU736947	EU339511	EU339474
Neofusicoccum parvum	CMW9081	AY236943	AY236888	AY236917
	CBS110301	AY259098	AY573221	EU673095

	1L87	KF778855	KF779045	KF778950
	WT1-1	MT252672	MT265236	MT265233
Neofusicoccum ribis	CBS 115475	AY236935	AY236877	AY236906
Neofusicoccum umdonicola	CBS 123645	MH863318	EU821874	EU821844
	CBS 123646	EU821905	EU821875	EU821845
Neofusicoccum viticlavatum	CBS 112878	AY343381	AY343342	KX465058
	CBS 110887	AY343383	AY343343	KX465061
Phyllosticta citricarpa	CBS 102374	FJ824767	FJ538371	FJ824778

6.3 Results

6.3.1 Field survey and isolations

Symptoms observed in the field included internal wood discoloration (Figure 1a, b), cankers, presence of exudate leaking out from bark wounds (Figure 1c), and general branch and shoot dieback (Figure 1d). Trees in the orchard appeared to be stressed, probably due to environmental factors and improper agronomic management; there were many insect injuries. An average of 3–5 portions of branches from each chosen tree were taken to the laboratory. All the symptomatic tissues and pycnidia found on the bark consistently yielded *Botryosphaeriaceae*-like fungi. Fungal isolates were stored at Dipartimento di Agricoltura, Alimentazione e Ambiente, Sezione di Patologia vegetale, University of Catania, and at Dipartimento di Scienze Agrarie e Forestali, University of Palermo.

6.3.2 Morphological identification and effect of temperature on mycelial growth rate

Observing pure cultures growing on PDA, a total of three different groups of *Botryosphaeriaceae*-like fungi, represented by almost 10 isolates each group, were separated. The isolate group WT1-

1 had floccose, fast-growing aerial mycelium, initially white/light grey be- coming darker with time. Isolates in the WT4-1 group had iron-grey mycelium with irregular margins, adpressed to the substrate, floccose but less aerial than isolates WT1-1. Isolates in the WT7-5 group had intermediate cultural characteristics between the other two groups (Figure 2 c,f,i). After almost 2 weeks growth, all three representative isolates (WT1-1, WT4-1, WT7-5) produced black pycnidia on pine needles, often covered by white mycelium (Figure 2 a,d,g). Conidia of the three isolates were 'fusicoccum-like', specifically, hyaline, aseptate and narrow (Figure 2b,e,h). Mean conidial length and width of isolates WT1-1, WT4-1 and WT7-5 were 15.1 and 6.1, 20.4 and 5.6, and 26.0 and 6.6 μm, respectively. All other measurements are reported in Table 2. All isolates exposed to different temperatures showed similar results in terms of minimum and maximum temperatures for growth, 5°C and 35°C, respectively. Temperature of 5°C resulted fungistatic for all the isolates, whereas 40°C resulted fungistatic for the isolate WT7-5 and lethal for WT1-1 and WT4-1. The optimum differed across species, with the highest value for *N. parvum* WT1-1, 29°C, 28.5°C for *N. mediterraneum* WT4-1, and 25°C for *B. dothidea* WT7-5 (Table 2).

6.3.3 <u>Molecular characterization and phylogenetic analysis</u>

For all representative isolates, PCR edited amplicons were approx- imately 523 bp long for the partial ITS region, 259 for *tef1*-α and 432 for *tub2*. The preliminary BLAST search resulted in high identity values (99%–100%) with the species confirmed in the phylogenetic tree. The ITS, *tef1*-α and *tub2* sequences generated were deposited in GenBank (Table 1). The partition-homogeneity test (p = .260) in- dicated no significant differences in the three genes dataset. The MP analysis showed that of 1,382 total characters (37 taxa), 330 were parsimony-informative, 127 parsimony-uninformative, 925 were constant, Tree length = 610, CI = 0.904918, RI = 0.966474 and RC =

0.874580. For ML analyses, the best-fit model of nucleotide evolution suggested GTR + I for ITS, HKY + I for *tef1*-α and HKY + G for *tub2*. The ML analysis showed that of 1,382 total characters, 925 were constant and 382 parsimony-informative. Isolate WT1-1 grouped with the clade of *N. parvum* (91/82%, MP and ML bootstrap support, respectively). The isolate WT4-1 grouped with *N. mediterraneum* clade (99/84), and the isolate WT7-5 grouped with the clade of *B. dothidea* (96/74) (Figure 3).

6.3.4 Pathogenicity on detached fruit

All tested isolates induced black lesions around the inoculation points on green walnut fruits. The infected area was rotted. All replicate inoculations caused the same symptoms (Figure 1 e–i). Controls had no lesions. Lesions caused by *N. parvum* WT1-1 were the most largest (lesion diameter mean = 5.1 cm), often able to colonize rapidly the entire pericarp, followed by *B. dothidea* WT7-5 (lesion diameter mean = 3.3 cm) and *N. mediterraneum* WT4-1 (lesion diameter mean = 2.5 cm). All three isolates colonized the inner layers of the pericarp. Re-isolations (~100%) confirmed the association between the inoculated fungus and symptoms observed on fruits.

6.3.5 Pathogenicity test on seedlings

All wounded plants formed necrotic lesions around the inoculation point under the bark (Figure 1l–n). Control plants had very superficial necrotic lesions corresponding with the wounds, but no evidence of lesion progression out of the inoculation point. All fungal isolates in- duced browning and wood discoloration around the wound. Lesions caused by *N. parvum* WT1-1 were the largest (length mean = 3.3 cm), followed by *N. mediterraneum* WT4-1 (length mean = 1.2 cm) and *B. dothidea* WT7-5 (length mean = 0.9 cm).

Table 2. Conidial and temperature measurements of *Botryosphaeriaceae* used in this study and comparison with previous studies.

Species	Isolate	Conidial size (μm)($L \times W$)	Mean $L \pm SD$	Mean $W \pm SD$	$L/W \pm SD$	Reference	Temp. Min	Temp. Opt.	Temp. Max
Botryosphaeria dothidea	WT7-5	(19.8–) 21.9–31.4 (–31.7) × (4.7–) 5.4–8.0 (–9.3)	26.0 ± 3.2	6.6 ± 0.9	4.0 ± 0.7	This study	5°C	25°C	35°C
	Type	(20–) 23–27 (–30) × 4–5 (–6)	24.7	4.9	5	Slippers et al. (2004)			
Neofusicoccum mediterraneum	WT4-1	(14.3–) 16.7–23.9 (–25.7) × (4.3–) 4.6–6.6 (–7.3)	20.4 ± 2.1	5.6 ± 0.6	3.7 ± 0.5	This study	5°C	28.5°C	35°C
	Type	(19–) 22–26 (–27) × (5.5–) 6 (–6.5)	24	6	4	Crous et al. (2007)			
Neofusicoccum parvum	WT1-1	(9.3–) 10.4–18.6 (–20.0) × (4.7–) 5.0–7.3 (–8.8)	15.1 ± 2.7	6.1 ± 0.8	2.5 ± 0.4	This study	5°C	29°C	35°C
	Type	(12–) 15–19	16.9	5.4	3.1	Slippers			

Figure 1. Symptoms of *Botryosphaeriaceae* on English walnut and pathogenicity tests. (a, b) Wood discoloration, (c) exudate leaking from bark wound, (d) dieback of walnut tree in the orchard, (e–i) pathogenicity tests on detached fruits, (e, f) external and internal husk rot by *Neofusicoccum parvum*, (g) *N. mediterraneum*, (h) *Botryosphaeria dothidea*, (i) control, (l–n) pathogenicity tests on seedlings, (l) lesion of *N. mediterraneum*, (m) *N. parvum*, and (n) *B. dothidea.*

Figure 2. Morphology of *Neofusicoccum parvum* isolate WT1-1, *N. mediterraneum* isolate WT4-1, *Botryosphaeria dothidea* isolate WT7-5. (a) Conidiomata of *N. parvum* on pine needles, (b) hyaline conidia, (c) 14-day-old culture on PDA incubated at 25°C, (d) Conidiomata of *N. mediterraneum* on pine needles, (e) hyaline conidia, (f) 14-day-old culture on PDA incubated at 25°C, (g) Conidiomata of *B. dothidea* on pine needles showing oozing of conidia, (h) mass of hyaline conidia and pycnidium, and (i) 14-day-old culture on PDA incubated at 25°C. Bars: a = 20 µm; b = 20 µm; d = 20 µm; e = 20 µm; g = 10 µm; h = 20 µm

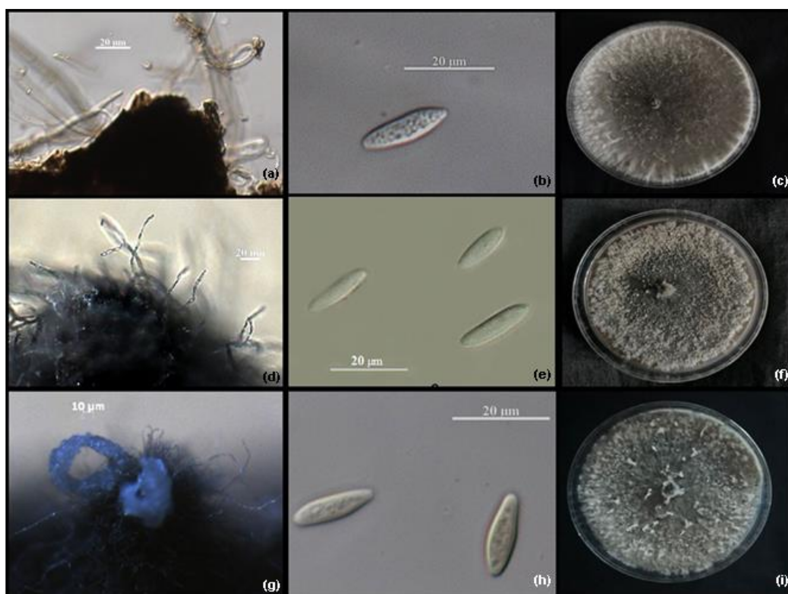
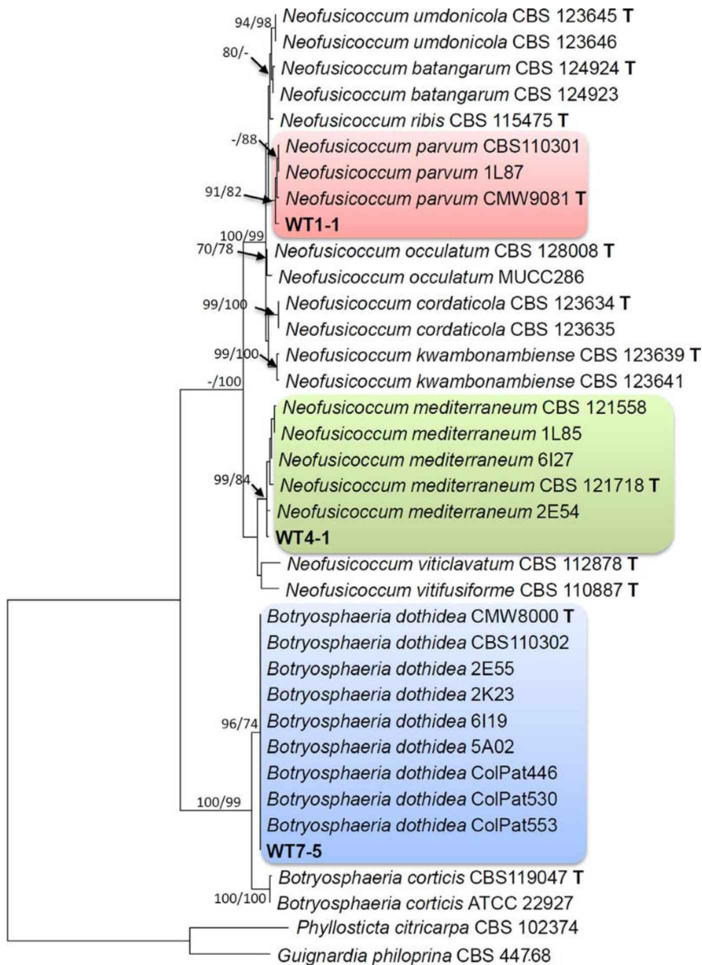


Figure 3. Single most parsimonious phylogenetic tree resulting from MP analysis of combined ITS, tef1-α and tub2 sequence data. Isolates in bold were sequenced in this study. Numbers represent bootstrap values (MP/ML). Values represented by '-' were <70%. Letter 'T' indicates type material. Scale bar represents the number of substitutions per site



6.4 Discussion

This work confirmed the association of three species of *Botryosphaeriaceae* causing cankers and other symptoms on walnut in Italy, including *B. dothidea*, *N. mediterraneum* and *N. parvum*. All these three species were tested on seedlings as well as on green fruits, demonstrating their pathogenic ability, although, based on the lesion lengths caused, they appeared to be of differing virulence. *Neofusicoccum parvum* was previously reported associated with walnut dieback in Spain, California, China and Korea (Chen, Morgan, Hasey *et al.,* 2014; Chen *et al.,* 2019; Cheon *et al.,* 2013; Inderbitzin *et al.,* 2010; López-Moral *et al.,* 2019; Yu *et al.,* 2015). In our study *N. parvum* induced the largest lesions. Similarly, in previous studies conducted in Spain and California, *N. parvum* was the most aggressive of several pathogens tested, regardless of the inoculated tissues. Many years ago in Italy, a report of walnut dieback attributed this symptom to *B. ribis* (Frisullo *et al.,* 1994), but no molecular sup- port was provided to present greater information within a group of taxonomically closely related species which are difficult to differentiate. Sakalidis et al. (2013) reported *N. parvum* from 90 hosts in 29 countries on six continents, considering it one of the widely distributed and most damaging species of *Botryosphaeriaceae*, characterized by a wide host range (host neutral; Phillips *et al.,* 2013; Slippers & Wingfield, 2007). In Sicily, the same pathogen was found attacking other crops such as fig (Aiello *et al.,* 2020), avocado (Guarnaccia *et al.,* 2016), mango (Ismail *et al.,* 2013), loquat (Giambra *et al.,* 2016), grapevine (Mondello *et al.,* 2013), and on the ornamental species *Rhaphiolepis indica* (Gusella *et al.,* 2019), confirming its wide host range. These data inform us about the need to properly and urgently monitor the spread of this serious plant pathogen.

The other two fungal species identified in our investigation were *N. mediterraneum* and *B. dothidea*, which were also reported on *Juglans* in other countries (Chen, Morgan, Hasey *et al.,* 2014; Li *et*

al., 2016; López-Moral *et al.,* 2019; Trouillas *et al.,* 2010), suggesting the contribution of these pathogens to disease development. Pathogenicity tests conducted in California showed that *N. mediterraneum* was intermediate in virulence between *N. parvum* and other *Botryosphaeriaceae*, including *B. dothidea* (Chen, Morgan, Hasey *et al.,* 2014). Since this first comprehensive research, Agustí-Brisach et al. (2019) investigated the biology of *N. mediterraneum* on English walnut, evaluating factors including temperatures, age and type of tissues in infection, and its interaction with *Diaporthe rushicola*, demonstrating that young shoots (one- to two-year-old) were more susceptible to the infection and colonization by the pathogen, com- pared to 3- to 4-year-old shoots.

The growth temperature tests confirmed that this group of fungi prefer warm temperatures, which combined stress to the trees (Mehl *et al.,* 2013), is an important factor in their ecology and epidemiology (Moral *et al.,* 2019). A deeper understanding of these factors in walnut production is crucial, especially in relation to *Botryosphaeriaceae*. For example, work in California revealed that pruning wounds in walnut remain susceptible to pathogen infection for at least 4 months after pruning in fall or winter, compared to pruning wounds in other woody crops (Michailides *et al.,* 2012), or the fact that spores of *Botryosphaeriaceae* may contaminate chain- saws as tissues bearing pycnidia are cut (T. J. Michailides, unpublished data/personal communication).

The discovery of these species causing diseases on walnut in Italy is an important result, not only for this emerging crop, but also for other nut or olive crops in the Mediterranean region. No *Botryosphaeriaceae* species seem to be specific to particular nut or olive hosts, but almost all plant pathogenic species in this family seem to be polyphagous (Moral *et al.,* 2019). In particular, *N. parvum*, *N. mediterraneum* and *B. dothidea* are known to infect almonds, olives, pistachios and walnuts (Chen, Morgan, Hasey *et al.,* 2014; Chen, Morgan, & Michailides, 2014; Gramaje *et al.,* 2012; Inderbitzin *et al.,*

2010; Lazzizera *et al.,* 2008; Urbez- Torres *et al.,* 2013). Often these crops are cultivated contiguously in Mediterranean Countries, allowing a flow of inoculum among these hosts (Moral *et al.,* 2019). In addition, as suggested by Lopes et al. (2016), it is crucial to monitor the pathways of introduction and dissemination of this group of fungi, not only in natural environments, but also in urbanized areas, where ornamental species may represent important inoculum sources. For most of these crops, in particular for walnut in Italy, new plantings design and the introduction of new varieties and new agronomic practices are changing scenarios in traditional cultivation; therefore, it is essential to understand how these pathogens could impact on yields. We are not excluding the possibility that further and wider surveys will reveal other species of *Botryosphaeriaceae* involved in diseases of walnut and other tree crops. Our study represented a preliminary and updated result on the association of some *Botryosphaeriaceae* species, such as *B. dothidea*, *N. mediterraneum* and *N. parvum* causing canker and dieback on walnut in Italy. Further investigations are required to better ascertain their incidence throughout the areas of cultivation, interactions amongst the various fungal species, difference in virulence be- tween pathogen species and disease progression in relation to different agronomic and environmental factors, in order to suggest proper control strategies.

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7. Other research activities on *Botryosphaeriaceae* diseases: Further Investigation on Limb Dieback of Fig (*Ficus carica*) Caused by *Neoscytalidium dimidiatum* in California

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7.1 Introduction

Fig (*Ficus carica* L.) acreage in the United States in 2017 reached 6,700 bearing acres producing 31,200 tons of fig (www.nass.usda. gov). Although the world's top three producer countries are Turkey, Egypt, and Morocco, the United States is in the top 10 (www.fao.org), with the state of California ranking first in the nation, accounting for nearly 98% of all U.S. figs produced (www.agmrc. org). The commercial cultivation of fig is an important crop, providing economic income to many Mediterranean countries and to the California fig industry, with the latter supporting research to improve the quality of the product, postharvest advanced technologies, and marketing (Crisosto et al. 2017).

Many diseases are reported to affect figs in California and

world- wide (Michailides 2003). Figs grown in California can suffer extensive losses due to fruit decay caused by fungi such as *Fusarium moniliforme* (endosepsis) (Michailides and Morgan 1998), *Aspergillus niger* (smut), *Alternaria*, and *Ulocladium* (Doster et al. 1996; Michailides 2003). In addition, canker diseases could represent a serious threat for fig growers, causing progressive yield losses over the years. In California, diseases caused by *Botryosphaeriaceae* spp. and *Diaporthaceae* spp. have been extensively investigated over the years (Chen et al. 2014a, b; Moral et al. 2019), revealing the presence of numerous taxonomic groups of species affecting different crops (Inderbitzin et al. 2010). These pathogens can induce severe symptoms, such as branch, shoot, and trunk cankers, and also blight fruits and leaves. Many reports of canker and dieback diseases on *Ficus* spp. have been published, showing *Botryosphaeriaceae* (Al-Bedak et al. 2018; El-Atta and Aref 2013; Mayorquin et al. 2012; Mohali et al. 2017) and *Diaporthaceae* (Hampson 1981; Lima et al. 2005; Re-hab et al. 2014) involved in these complex diseases. In addition to other *Ficus* spp., the cultivated fig is also attacked by botryosphaeriaceous and diaporthaceous fungi worldwide (Aiello et al. 2020; Banihashemi and Javadi 2009; Çeliker and Michailides 2012; Javadi and Banihashemi 2005). Among members of the *Botryosphaeriaceae* family, *Neoscytalidium dimidiatum* was reported in several countries to cause cankers and dieback on different *Ficus* spp., including the common fig (Al-Bedak et al. 2018; Elshafie and Ba-Omar 2002; Giha 1975; Mirzaee et al. 2002; Ray et al. 2010). This species produces two different asexual states known as synanamorphs: the coelomycetous morph, producing pycnidia with conidia (*Fusicoccum*-like), and the hyphomycetous morph, producing powdery arthric chains of conidia (*Scytalidium*-like conidia or arthrospores) (Farr et al. 2005; Nattrass 1933; Sutton and Dyko 1989), which is the reason why it has been characterized by a restless taxonomic process, going through different names and descriptions (*Torula dimidiata*, *Hendersonula toruloidea*, *Nattrassia mangiferae*, *Scytalidium dimidiatum*, *Scytalidium hyali-*

num, *Fusicoccum dimidiatum*, *Neoscytalidium hyalinum*). In 2006 Crous et al. (2006) established the new genus *Neoscytalidium* (Crous and Slippers). In California, this species was reported with the old name *Hendersonula toruloidea* on fig (Paxton et al. 1964; Warner 1952), walnut (Wilson 1949), citrus (Calavan and Wallace 1954), and recently as an emerging pathogen on citrus, grape, and almond (Mayorquin et al. 2016; Nouri et al. 2018; Rolshausen et al. 2013). Furthermore, symptoms of a canker disease on 'Kadota' figs in California were also reported in the early 1950s, associated with *Phomopsis* species (English 1951, 1952; Hansen 1949). However, the problem seemed to gradually fade away, probably because acreage of 'Kadota' has decreased significantly over the years. In the last several years, figgrowers in California have noticed a large number of trees in many or- chards losing large limbs because of severe dieback. On the basis of previous reports, and to elucidate the limb dieback disease of fig in California, we conducted several experiments with the following aims: to ascertain the incidence of the disease and the role of *N. dimidiatum* in the limb dieback etiology, to study the influence of environmental and agronomic factors in the disease etiology, and to evaluate the susceptibility of different fig cultivars to the disease in California.

7.2 Materials and Methods

7.2.1 Field survey and fungal isolations.

A total of 16 fig orchards located in Fresno, Kern, and Madera Counties (central and southern San Joaquin Valley in California) were surveyed for 3 years (2005 to 2007), collecting every year about 10 to 15 branches and shoots showing symptoms of cankers and dieback from five different cultivars, including 'Black Mission', 'Calimyrna', 'Conadria', and also a Stanford caprifig (male tree) and another

unknown caprifig cultivar. Symptomatic tissues were surface disinfected with household bleach at 10% (vol/vol) in sterile water for 3 min. Small pieces (3 to 5 × 2 to 5 mm) from the margins of cankers were cut with a sterile scalpel and placed in Petri dishes containing 2% potato dextrose agar (PDA) acidified with lactic acid (2.5 ml of 25% [vol/vol] per liter of medium; APDA) to minimize bacterial growth. Petri dishes were incubated at 25 ± 3°C for 2 to 7 days, until fungal colonies were large enough to be examined. Occasionally tissue from stained wood segments distant from the canker margin, healthy-appearing wood away from the canker, infected lenticels or growth cracks, and tissue from insect borings were plated out. *N. dimidiatum* colonies were transferred to APDA dishes to obtain pure cultures, and single spore isolates were then stored in our collection. Recovered isolates used for further investigation are maintained in the culture collection of the Department of Plant Pathology at the University of California, Davis (Kearney Agricultural Research and Extension [KARE] Center in Parlier).

7.2.2 Effect of different temperatures on mycelial growth of N. dimidiatum.

To determine the cardinal temperatures of growth, a 4.76-mm plug of a 3-day-old colony of the isolate of *N. dimidiatum* 2D3 was removed and transferred to the center of 90-mm Petri dishes of APDA and incubated at eight different temperatures, from 5 to 40°C. Four Petri dishes were used for each temperature as replicates. The experiment was repeated once. After 3 days of incubation, the largest and smallest diameters of colonies were measured with a digital scale ruler. Mean data were converted to radial growth (in mm). Data from two experiments were combined after checks for homogeneity of variances via *F* test. A nonlinear adjustment of the data was applied via the generalized Analytis Beta model, as described by Lòpez-Moral et al. (2017), and the optimum growth temperature was calculated

according to the formula provided by the same authors.

7.2.3 Pathogenicity tests on detached shoots.

Preliminary pathogenicity tests were conducted on detached shoots collected from an experimental orchard at the KARE Center under laboratory conditions. Current season shoots ('Calimyrna'), 15 to 25 cm long, were surface sterilized for 4 min in a dilute mixture of bleach and alcohol (160 ml of 5.25% NaOHCl bleach and 160 ml of ethanol/10 liters of water) and allowed to air dry on plastic screens in plastic rectangular chambers of 30 × 23 × 10 cm. A 7-mm in diameter plug from 14-day-old cultures of *N. dimidiatum* (isolates 3C02, 3C07) grown on APDA was used to inoculate each shoot. Wounds were made with a 7- mm cork borer, and the mycelial plug was placed on each wound upside down and covered with semitransparent film (Parafilm®) to prevent desiccation. Water was then added to the bottom of the plastic container to create a humid environment, and the containers were incubated at 30°C. The experiment consisted of four treatments: wounded shoots noninoculated, nonwounded and inoculated, wounded and inoculated, and nonwounded and noninoculated. Ten shoots per treatment served as replicates. The experiment was repeated once with a slight modification, using 4-year-old shoots instead of current shoots. Presence of cankers (disease incidence) and length of cankers were recorded 40 days after the first experiment and 30 days after the repetition.

7.2.4 Pathogenicity test in the field.

Based on the isolation results, pathogenicity tests in the field were conducted with *N. dimidiatum* (isolate 2D03) and *Phomopsis* sp. previously isolated from a symptomatic 'Calimyrna' fig limb in Madera County. These two species were inoculated onto 2-year-old

shoots on 10 ‘Calimyrna’ trees in the south row of an experimental orchard located at the KARE Cen- ter. A total of 15 shoots per treatment were used. Half of the shoots were tied to a string attached to a plastic bag filled with dried soil as a weight to bend the shoots at a 45° angle and expose them more to direct sunlight to induce sunburn. The remaining shoots were left alone and were not exposed to direct sunlight because they were shaded by the foliage above them. A cork borer of 7-mm diameter was used to create a wound, and a mycelial plug of 7 mm of each fungus was used to inoculate each shoot. The wounds and the mycelial plugs were wrapped with semi-transparent film to prevent desiccation. Control consisted of a sterile plug of PDA. The length of canker at either side of the inoculation was recorded after 1 year and 4 months. Reisolation from the margins of the cankers was done to fulfil Koch’s postulates.

7.2.5 Effect of summer and winter pruning on infection development.

To study the effect of summer and winter pruning on infection development, trees of the ‘Calimyrna’ experimental orchard at the KARE Center were pruned and inoculated in August and February in a spore suspension at a concentration of 1×10^5 spore/ml of *N. dimidiatum* isolate 3B02. Inoculations were made on both summer and winter pruning cuts at different times after pruning, at 0, 1, 2, 3, 4, 5, 6, 7, 14, and 21 days after pruning, using 10 shoots per each treatment. These serial inoculations were performed to observe differences in the length of the lesions and then evaluate the period of susceptibility of pruning cuts. Pruned but noninoculated shoots served as controls. After the inoculum was sprayed on the pruning cut, the inoculated wounds were covered with semitransparent film to prevent desiccation. Length of the cankers was recorded twice: 1 and 2.5 years later for the summer pruning and 6 months and 2 years later for the winter pruning.

7.2.6 *Effects of stress treatments on infection development.*

The effects of different environmental and agronomic stresses on the infection development were evaluated. Specifically, the effects of sunburn and mechanical injuries were studied. Eight treatments were conducted in this experiment: shoots wounded with mallet and inoculated; shoots wounded with mallets, sunburned, and then inoculated; shoots wounded only with mallets and noninoculated; shoots sunburned and then inoculated; shoots inoculated but nonwounded (control 1); shoots wounded but noninoculated (control 2); shoots wounded, inoculated, and painted with whitewash (Surround®, Nova-Source, Tessenderlo Kerley, Inc., Phoenix, AZ, U.S.A.); and shoots painted with whitewash and then inoculated. Large limbs (3 to 4 years old) of 15-year-old 'Calimyrna' trees located at the KARE Center were subjected to these stress treatments in August. To sunburn or heat the shoots above ambient conditions, portions of the shoots were wrapped with black plastic (Fig. 1E). To simulate wounding by mallets, shoots were wounded with the threaded end of a 7.94- × 304.8-mm (5/16 × 12-inch) carriage bolt. To cool them below ambient conditions, shoots were painted with a white tree trunk paint. In this experiment, 10 replicates were used for each treatment, for a total of 10 trees. Three weeks after the beginning of each treatment, the bark was removed with a 7-mm cork borer, and the shoots were inoculated with a 7- mm mycelial plug (isolate 3C02). Symptoms were recorded 2 and a half years after the inoculations.

7.2.7 *Susceptibility of fig cultivars to the infection.*

To evaluate the susceptibility of various fig cultivars to the limb dieback, 'Brown Turkey', 'Black Mission', 'Calimyrna', 'Conadria', 'Kadota', and 'Sierra' were planted in an experimental orchard at the KARE Center and used for the experiment. A total of eight trees per cultivar were used, and two 1-year-old shoots per tree were inoculated

with a 7-mm mycelial plug from a 14-day-old colony of the isolate 3C02 placed on wounds made with a 7-mm cork borer. Inoculations were performed in September, and results (length of cankers) were recorded twice, 2 months and 1 and a half years after the inoculations.

7.2.8 Cankers eradication and pathogen recovery.

Existing cankers of different inoculation experiments were pruned 5.08 cm (2 inches) below the canker margin from half of the trees in the "effect of summer and winter pruning on infection development" experiment and from half of the trees in the "effects of stress treatments on infection development" experiment. Two years later the cankers from pruned and unpruned shoots were measured to see whether canker removal can contain pathogen movement in the shoots. Reisolations were made on acidified PDA from shoots subjected to this investigation to ascertain the possible recovery of *N. dimidiatum.*

7.2.9 Data analysis.

Data of this study were analyzed in SAS (release 9.3; SAS Institute Inc.) and Statistix 10 (Analytical Software 2013). Data were tested for normality and homogeneity of variances, and then the analysis of variance was performed. Mean differences were compared with Fisher's least significance difference test at $P = 0.05$.

7.3 Results

7.3.1 Field surveys, fungal isolations, symptoms, and signs of the disease.

Results of the isolations from all 16 orchards surveyed in 3 years consistently showed the presence of *N. dimidiatum*, in both symptomatic and asymptomatic samples. Close examination of collected samples from trees with limb dieback revealed that the pathogen produced both arthrospores and pycnidia in woody tissues. Arthrospores are loose and develop in the space between the bark and the woody tissues from mycelia of the fungus that break into short pieces (Fig. 1D). Pycnidia were found embedded in the bark (Fig. 1C) and in general produced light-colored, unicellular pycnidiospores. The second most common fungus isolated from these surveys was *Phomopsis* spp., although its incidence seemed to decrease during this study. *Phomopsis* spp. incidence was surprisingly high (100%) in the second year of survey in one orchard of cultivar 'Black Mission' (Table 1). Symptoms observed in the field included internal wood discoloration, branch canker and dieback, signs (pycnidia) of the pathogen in the bark, and arthrospores under the bark (Fig. 1A to 1D). The following isolates of *N. dimidiatum* were used in this study: 2D3/2D03, 3C2/3C02, 3C7/3C07, and 3B02. Because of the uniformity and similar growth characteristics of these isolates, three random isolates, 2D3, 3C2, and 3C7, were molecularly identified on the basis of six loci in a study by Inderbitzin et al. (2010).

7.3.2 *Effect of different temperatures on N. dimidiatum mycelial growth*.

After 3 days of incubation, no mycelial growth was observed at 5 and 10°C. Mycelial growth was observed at all other temperatures, showing different mean values: 7.0 mm at 15°C, 11.1 mm at 20°C, 36.3 mm at 25°C, 39.6 mm at 30°C, 38.0 mm at 35°C, and 3.5 mm at 40°C. Optimum growth occurred at 31.5°C (Fig. 2).

7.3.3 Pathogenicity tests on detached shoots.

Pathogenicity tests con- ducted under laboratory conditions on detached current shoots, and 4-year-old detached shoots in experiment 2, showed that *N. dimidiatum* causes canker on fig tissues. Specifically, in experiment 1, three statistically different groups emerged from the analysis. Results with isolate 3C7 on wounded shoots are significantly different from those of all other treatments, showing the largest cankers. Inoculations with isolate 3C2 on wounded and unwounded shoots, and 3C7 on un- wounded shoots, did not reveal statistically significant differences, showing an intermediate level of canker length. However, treatments with wounded and unwounded shoots not inoculated did not produce lesions, showing a separate statistical group. In both experiments the highest incidence (100%) was recorded on wounded shoots inoculated with either isolate. In experiment 2, treatment with isolate 3C7 on wounded shoots produced the longest cankers, as it did in experiment 1, followed by treatment with isolate 3C2 on wounded shoots, and the shortest lesions with the same isolate on unwounded shoots. Interestingly, isolate 3C7 inoculated on unwounded shoots did not produce lesions this time, and the incidence of infection was 0%. In experiment 2, the isolates induced smaller lesions than those in experiment 1 (Fig. 3). Arthrospores were abundant under the bark on these shoots, as were pycnidia with mature pycnidiospores.

7.3.4 <u>Pathogenicity test in the field.</u>

Pathogenicity tests in the field conducted on 2-year-old shoots with *N. dimidiatum* (2D03) and an isolate of *Phomopsis* sp. revealed significant differences between the two species. *N. dimidiatum* induced lesions of 24.8 mm (no sun- burn) and 19 mm (sunburn), but *Phomopsis* sp. did not induce any lesions significantly larger than the discoloration that resulted from the control (11.9 mm vs 10.9 mm) (Table 2). This test in the field was performed also to determine effects of sunburn on the shoots inoculated with *N. dimidiatum*. In this

experiment, there were no significant differences between the two treatments. Because maximum air temperature reached 32.2°C on only 4 days during the shoot inoculation experiment, the heat stress may not have been sufficient to show an effect (Table 2). However, because we observed that cankers were often associated with sunburned tissues, the hypothesis that sunburn affects *Neoscytalidium* canker was investigated separately in another experiment.

7.3.5 Effect of summer and winter pruning on infection development.

Results from summer and winter pruning inoculations revealed significant differences between the two seasonal treatments. Cankers from summer pruning were significantly longer than those that occurred after winter pruning. Lesions were measured twice, at two different times, and in both cases significant differences were confirmed. In both evaluations the average length of summer lesions was 28.5 cm, com- pared with 9 cm for the winter treatment. Very small lesions in the last treatments (21 days after pruning) were observed from the serial inoculation time after pruning, suggesting that pruning cuts become less susceptible after 3 weeks (data not shown).

7.3.6 Effects of stress treatments on infection development.

This experiment was performed to see whether environmental or agronomic stresses could affect infection establishment and development. Limbs wounded with mallets and also sunburned had the largest cankers among all the treatments. Intermediate length of cankers was recorded for limbs only wounded with mallets or only sunburned, followed by limbs only wounded with mallets or only sunburned without fungal inoculation. No significant differences were recorded between the cankers on whitewashed limbs before and after inoculation and the nonwounded but inoculated control (Fig. 4).

7.3.7 Susceptibility of fig cultivars to the infection.

The first symptom evaluation, conducted 2 months after the inoculation of the six fig cultivars, revealed two statistically different groups, with cultivars 'Sierra', Kadota', and 'Black Mission' being more susceptible than cultivars 'Brown Turkey', 'Conadria', and 'Calimyrna'. The second evaluation, performed a year and a half later, confirmed the results of the first evaluation, with the exception of cultivar 'Black Mission', which showed moderate susceptibility between the less susceptible 'Brown Turkey', 'Conadria', and 'Calimyrna' and the more susceptible 'Kadota' and 'Sierra' (Fig. 5).

7.3.8 Canker eradication and pathogen recovery.

Pruning the shoots 5.08 cm below the obvious external margins of cankers effectively contained pathogen movement within the shoots. Isolations from the cut surface of pruned shoots from trees in both experimental orchards at the KARE Center produced no *N. dimidiatum*. Otherwise, the fig canker pathogen was consistently recovered from the margins of cankers in unpruned shoots that remained on the trees in this or- chard (frequency average = 43%)

Table 1. Incidence of fungal species emerging from 3 years of surveys in Fresno, Madera, and Kern counties.

Cultivar	Month	*Neoscytalidium dimidiatum*	*Phomopsis* sp.	*Botryosphaeriaceae* sp.	Year
Black Mission	May	26.4	66.4	1.0	1
Calimyrna	May	78.3	13.3		1
Conadria	June	82.2	7.8	7.8	1
Calimyrna	June	65.5	32.2	3.3	1
Black Mission	June	11.3	55.5	1.7	1
Black Mission	June	6.7	93.3		1
Calimyrna	May	75	17		2
Black Mission	May	59	0		2
Black Mission	May	8	100		2
Conadria	May	86	0		2
Caprifig	May	85	0		2
Conadria	May	99	0		2
Black Mission	May	97	17		2
Black Mission[w]	May	90	0		2
Black Mission[x]	May	100	0		2
Stanford caprifig	May	90	3		2
Calimyrna	May	100	0		2
Calimyrna	May	85	10		2
Calimyrna	May	100	0		2
Calimyrna	May	32	39		3
Black Mission	June	84[y]	0		3
Black Mission	Novemb.	100[z]	0		3

[w] Isolations from infected lenticels or growth cracks.
[x] Isolations from tunnels of boring insects.
[y] *N. dimidiatum* was isolated from 68% of the dark brown staining of the woody tissues in advance of the cankers.

Table 3. Pathogenicity test in the field.

Treatment	Species	Canker length (mm)
Sunburn	*Neoscytalidium dimidiatum*	19.0 a[z]
Non-sunburn	*N. dimidiatum*	24.8 a
	Phomopsis sp.	11.9 b
	Control	10.9 b

[z] *N. dimidiatum* was also isolated from 15% of symptomless shoots

Figure 1. Effect of temperature on mycelial growth of isolate D3 of *Neoscytalidium dimidiatum*. The averages of radial growth and temperature were adjusted to a nonlinear regression curve via the Analytis Beta model. Y represents standardized radial growth. Data points are the means and vertical bars are the standard error of the means.

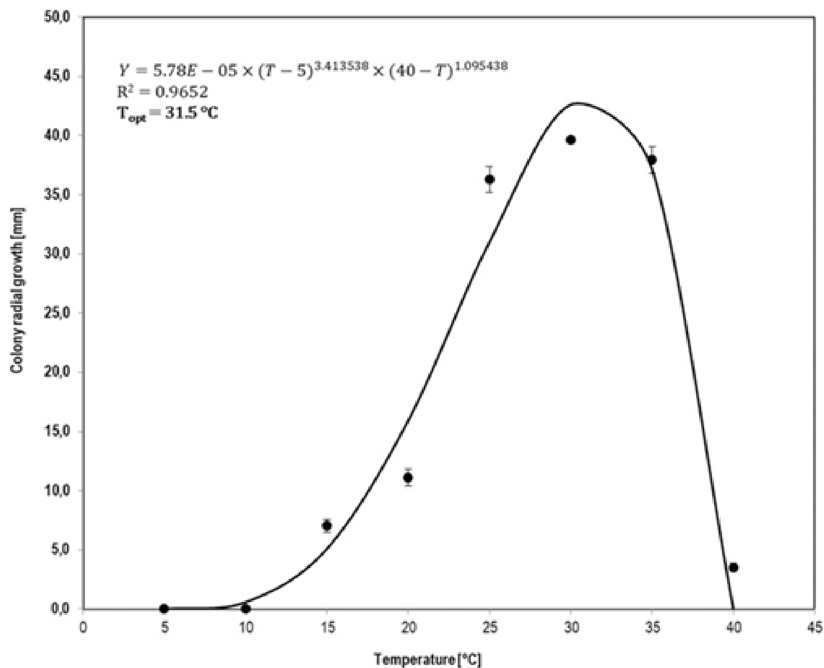


Figure 2. Symptoms and signs of *Neoscytalidium dimidiatum* on fig. A, Limb dieback of cultivar 'Black Mission' in the field. B, Internal wood discoloration of cultivar 'Black Mission'. C, Pycnidia observed on fig shoot. D, Arthrospores developed under the bark. E, Shoot wrapped with black plastic to create the sunburn treatment.

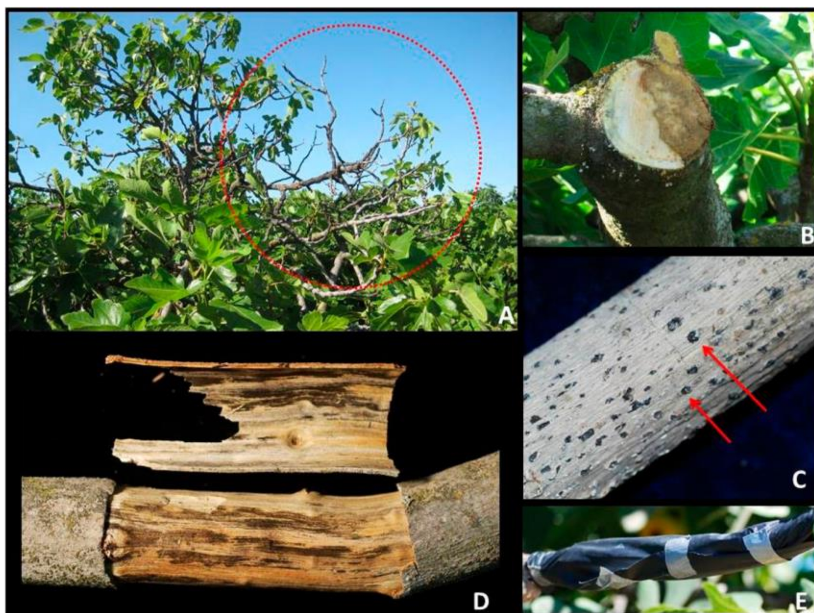


Figure 3. Pathogenicity of *Neoscytalidium dimidiatum* on detached fig shoots. Average lesion length resulting from inoculation with a mycelium plug of *N. dimidiatum* onto current and 4-year-old shoots. Percentages above the columns indicate the incidence of infection. Letters above the columns indicate treatments significantly different ($P < 0.05$). Letters refer to the specific experiment.

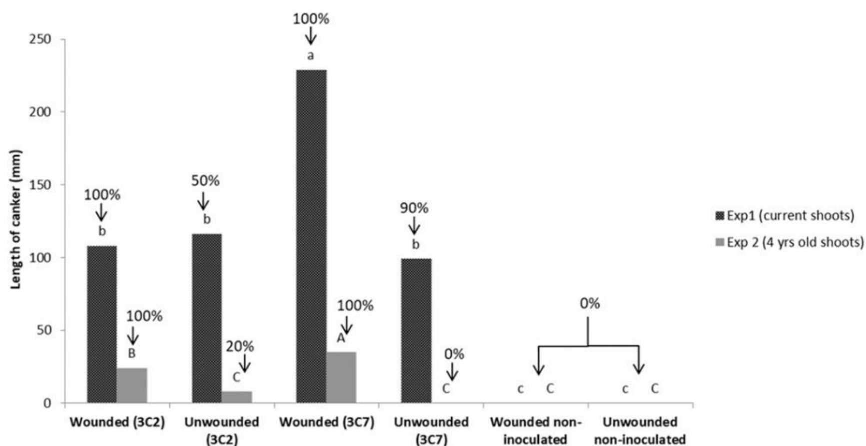


Figure 4. Effects of different stress factors on lesion length after inoculation of fig shoots with *Neoscytalidium dimidiatum*. Different letters above columns indicate significantly different treatments ($P < 0.05$).

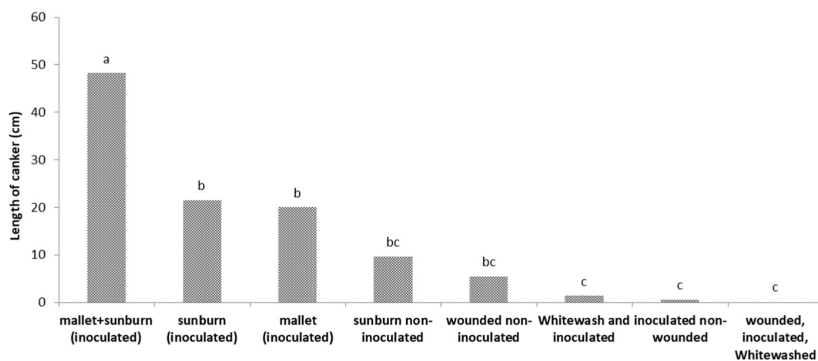
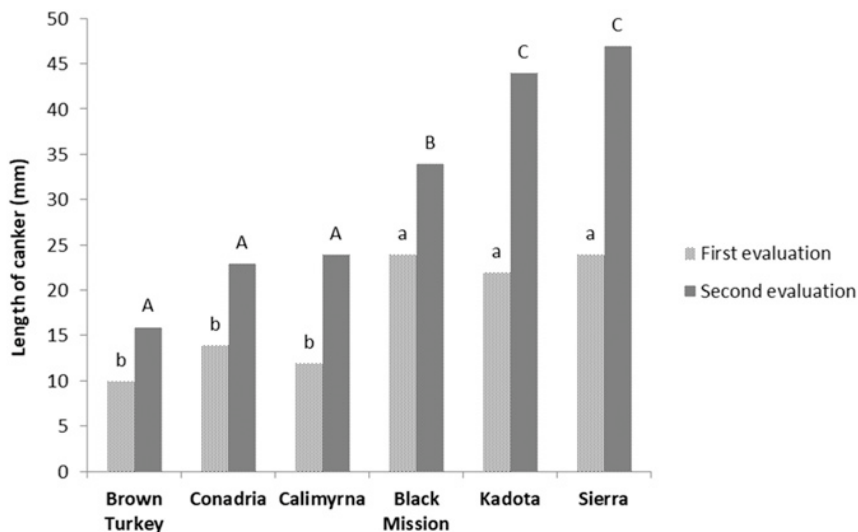


Figure 5. Cultivar susceptibility (as indicated by lesion length) to limb dieback disease caused by *Neoscytalidium dimidiatum*. Different letters above columns indicate significantly different treatments (P < 0.05). Letters refer to specific (first and second) disease evaluation.



7.4 Discussion

The present study investigated the etiology and epidemiology of fig limb dieback in California. Results from our surveys conducted in the main fig production counties of California reveal that the two most common species isolated from cankers of symptomatic branches and shoots were *N. dimidiatum* and *Phomopsis* spp. *Phomopsis* has been reported in other countries as an important fig canker pathogen. In Iran, its association with fig canker has been known for >25 years (Banihashemi, unpublished data). In California, *Phomopsis cinerascens* was reported in 1936 as an epidemic pathogen on the cultivar 'Kadota' (Ferguson et al. 1990). Phomopsis canker can be found in all commercial fig cultivars, but it is most devastating in 'Kadota' (Obenauf et. al. 1978). However, pathogenicity tests conducted in this study using both *N. dimidiatum* and *Phomopsis* spp. revealed that *N. dimidiatum* induced lesions, unlike *Phomopsis*. 'Kadota' probably was susceptible to *Phomopsis* infection during those years, and its progressive displacement with other cultivars, perhaps less susceptible, led to a decrease in the incidence of this pathogen over the years. When the surveys of orchards with putative canker diseases were performed, no commercial orchards of 'Kadota' were located in counties of the central San Joaquin Val- ley in California. The 3-year surveys of other fig cultivars showed high levels of both *N. dimidiatum* and *Phomopsis* spp. only in the first year, an indication that these pathogens could co-occur in the same canker tissues. However, in survey years 2 and 3, with the exception of one 'Black Mission' orchard where all samples produced *Phomopsis* spp., *Phomopsis* spp. were not isolated or were isolated at low levels in a few orchards (Table 1). Obviously, the incidence of *Phomopsis* spp. was reduced in years 2 and 3 from surveyed symptomatic tissues. In contrast, the incidence of *N. dimidiatum* was high in most samples during the 3-year survey. However, recent isolations have shown the presence of *Phomopsis* spp. associated with symptomatic samples,

although at low percentage compared with *Neoscytalidium* frequency. Therefore, we can affirm that *Phomopsis* spp. is in somehow associated with the disease, but additional investigations are needed to confirm the interactions between these two species and disease development.

N. dimidiatum has been reported worldwide, causing diseases on many other important crops (Derviş et al. 2019; Polizzi et al. 2009; Rolshausen et al. 2013; Türkölmez et al. 2019) and in the study of Marques et al. (2013) it was considered the most aggressive of all *Botryosphaeriaceae*. A recent study conducted in California on almond branch and trunk cankers showed a high incidence of *N. dimidiatum* and confirms that this disease appeared to be widespread, suggesting a recent increase of this pathogen and the diseases it causes (Nouri et al. 2018). In California, the presence of different susceptible crops in contiguous areas allows an easy flow of inoculum (arthrospores) between tree species (Moral et al. 2019). Our optimum growth temperature results accord with those of Nouri et al. (2018), showing an optimum temperature of 31.5°C. In this study we found a significant difference in lesion length between trees pruned in summer and those pruned in winter. Lesions from summer pruning were always significantly longer than those on winter-pruned trees. Also, winter pruning is less problematic than summer pruning, when more severe infections and higher inoculum levels can occur. These results demonstrate that summer temperatures are an important factor for the development of infection by *N. dimidiatum*, as stated by Hassan et al. (2011) and Sadowsky et al. (2007). In fact, both reports showed the effect of heat stress treatments as a predisposing factor to *N. dimidiatum* infection. In Israel, severe symptoms on grapefruits appeared after extremely hot and dry weather events for several consecutive days (Oren et al. 2001). In Oman, infection on *Albizia lebbeck* occurred in the summer of 1998 when the average temperature was 40°C (Elshafie and Ba-Omar 2002), as was observed in Iraq on different hosts (Hassan et al. 2009). Our results accord with previous research of

English et al. (1975), who described the canker pathogen *H. toruloidea* in California as extremely temperature sensitive, able to cause appreciable infection only in summer. However, a recent study conducted in California on almond showed that *N. dimidiatum* isolates infected almonds regardless of the month of inoculation, although the authors affirmed that the winter of 2015 was particularly dry and warm (Nouri et al. 2018). Many authors refer to *N. dimidiatum* as a weak or opportunistic pathogen, invading tissues through wounds and openings, especially when the host is stressed. Schoeneweiss (1975) suggested that stress plays a key role, exerting its most pronounced effect by predisposing plants to facultative parasites, especially weak or nonaggressive pathogens. In this study, experiments conducted with different stress treatments demonstrated that the combination of mechanical injuries (mallet) and sunburn led to the longest cankers, followed by lesions derived from inoculations on wounded shoots (mallet) or only sunburned shoots. In this experiment, and in the pathogenicity tests on 4-year-old detached shoots, wounds seemed to be necessary to initiate the infection process. However, in pathogenicity tests on current shoots, the infection occurred also on unwounded ones, probably because the tissues were not lignified enough and the pathogen was able to penetrate without a pre-existing wound. Similarly, in a study conducted on a dragon fruit canker disease caused by *N. dimidiatum*, Fullerton et al. (2018) concluded that the tissues most susceptible to infection were the tips of rapidly growing cladodes. Mature cladodes were highly resistant to infection. Davison (1972) showed that *H. toruloidea* can infect an unwounded tree at the ideal temperature of 25°C, but wounding resulted in the higher disease incidence, supporting our results by suggesting that injuries such as sunburn, pruning wounds, and other mechanical injuries (i.e., mallet) are crucial in the infection process, as was also shown by other authors (Calavan and Wallace 1954; Nouri et al. 2018; Oren et al. 2001). Our results also showed that pruning wounds are susceptible to infection for 3 weeks. The same experiment revealed

that shoots treated with whitewash and inoculated did not produce lesions. Treatments with whitewash seem to be effective at both protecting trees from sunburn, thus preventing development of cracks and other wounds on shoots, and suppressing infection by the pathogen and canker formation.

In this investigation, six commercial fig cultivars were evaluated for their susceptibility to canker formation by *N. dimidiatum.* Among these cultivars, 'Brown Turkey', 'Conadria', and 'Calimyrna' (non- persistent figs, i.e., needing pollination to keep the fruit) are less susceptible than cultivars 'Kadota' and 'Sierra', which were more susceptible, with the cultivar 'Black Mission' showing moderately susceptibility. Interestingly, these last three cultivars are persistent (i.e., they do not need pollination for fruit to develop). At the end of all experiments, we ascertained that pruning 5 cm below the canker could successfully remove the pathogen from the infected shoots. The pathogen was never recovered from shoots that were pruned 5 cm below the canker, an indication that the pathogen has difficulty advancing more than 5 cm internally beyond the external canker mar- gin. This information should be used by growers as a best practice to safely remove cankers from their fig orchards. This investigation rep- resents a contribution to our understanding of this destructive emerging pathogen in California fig orchards, and future research should aim at developing efficient control strategies.

7.5 ***References***

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8. Other research activities on *Botryosphaeriaceae* diseases: Characterization of *Neofusicoccum parvum* causing canker and dieback on *Brachychiton* species

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8.1 Research

Brachychiton Schott & Endl. is a small genus with about 30 species native to Australia, initially included in *Sterculia*, later separated as distinct genus (Guymer, 1988). Because a distinctive shape of the trunk of some species, this genus is well-known as the "bottle tree" (Thabet *et al.,* 2018). Members of the *Brachychiton* genus were used as food by Australian Aborigines, some are used as ornamental trees or shrubs, and many members of the genus are used for medical purposes (Rao, 1991; Salem *et al.,* 2014; Thabet *et al.,* 2018).

In Sicily (Italy) *Brachychiton* species are present in the territory for their aesthetic value, being used as urban and ornamental trees (Bazan *et al.,* 2005; Scafidi *et al.,* 2016). The phytopathological situa-

tion of this genus seems to be outdated. Concerning the two species of Brachychiton investigated in this study, including *B. acerifolius* (A. Cunn. ex G. Don) Macarthur & C. Moore) and *B. populneus* (Schott & Endl.) R. Br., fungi belonging to *Armillaria, Athelia, Amphitiarospora, Diplodia, Discosia, Dothiorella, Gloeosporium* and *Phyllosticta*, as well as many *Phytophthora* spp. have been sporadically reported (Farr & Rossman, 2021). During the autumn of 2020, a survey conducted in an ornamental nursery in Catania Province (Sicily), revealed the presence of 5 to7- years-old trees of *B. acerifolius* and *B. populneus* showing cankers, gummosis, wood discoloration and dieback. Due to the aggressiveness of the causal agent, the possibility of host jump, and the novelties of these symptoms undescribed for this genus, the aim of this study was to identify and characterize the causal agent and to test its pathogenicity.

Surveys were conducted in January of 2021 in a commercial nursery located in Catania Province, Sicily, Italy. Symptomatic samples were collected and brought to the laboratory of the Dipartimento di Agricoltura, Alimentazione e Ambiente, University of Catania for further investigations. Small sections of symptomatic tissues (discoloured trunk) 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 sulfate (Sigma-Aldrich, St. Louis, MO, USA) to pre- vent bacterial growth, and then incubated at 25 ± 1 °C for 3—5 days. To study conidial morphology, sporulation of three representative isolates was induced by transferring isolates onto Technical Agar (AT, 1.2% Agar, Biolife, Milan-Italy) with sterilized pine needles, and incubating at room temperature. After two weeks, pycnidia were mounted in lactic acid and 50 conidia were measured using a fluorescence microscope Olympus-BX61 (Olympus, Tokyo, Japan) coupled to an Olympus DP70 digital camera; images and measurements were captured using the software analySIS 3.2 (Soft Imaging System GmbH, Münster,

Germany) and DP Controller 1.1.1.89 (Olympus Optical Co., LTD). Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). The internal transcriber spacer region (ITS) of the nuclear ribosomal RNA operon was amplified with primers ITS5 and ITS4 (White *et al.,* 1990), the primers EF1-728F and EF1-986R (Carbone & Kohn, 1999) were used to amplify part of the translation elongation factor 1alpha gene (EF-1α), and primers set Bt2a and Bt2b (Glass & Donaldson, 1995) were used for the partial beta-tubulin (*tub2*). The PCR products were sequenced in both directions by Macrogen Inc. (Seoul, South Korea). The DNA sequences generated were analyzed and consensus sequences were computed using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar *et al.,* 2018), and submitted to GenBank. The sequences obtained in this study were subjected to BLASTn queries in NCBI and the curated database TrunkDiseaseID. org (Lawrence *et al.,* 2017) to determine the closest relatives. Multiple sequences alignment was conducted using MEGA X. A total of 39 taxa were considered in the analysis according to recent literature (Bezerra *et al.,* 2021), and *Botryosphaeria dothidea* isolates CBS 110302 and CMW8000 served as the outgroups. Phylogenetic analyses was conducted on a concatenate data- set (ITS + EF-1α + *tub2*), based on Maximum Parsimony (MP) using PAUP v.4.0a (Swofford, 2003). MP parameters were set as follows: heuristic search function and tree bisection and reconstruction (TBR) as branch swapping algorithms with the branch swapping option set on “best trees” only. Gaps treated as “missing”, the characters unordered and of equal weight and Maxtrees limited to 100. MP scores (TL, CI, RI, RC, and HI) were calculated. A total of 1,000 bootstrap replicates were performed to test the robustness of the tree topology. Pathogenicity tests were conducted on six potted, healthy, 5-years old plants in the nursery (out-door), three *B. acerifoilius* and three *B. populneus* respectively and on four 3-years-old plants under laboratory conditions (indoor) (*B. populneus*). A piece of bark was removed with

a 7.5-mm-diam. cork borer, and 7.5-mm-diameter mycelial plugs taken from a 4-day- old culture were applied to three spaced wounds on each plant. Wounds were covered with Parafilm® (Pechney Plastic Packaging Inc., Chicago, USA) to pre- vent desiccation. Controls consisted of sterile PDA plugs applied to wounds (three plants). The presence and length of the resulting lesions of the 5-years-old plants were recorded ten weeks after the inoculation, whereas 17 days after the inoculation for the 3-years- old plants. Survey in the nursery revealed symptomatic plants showed cankers, subcortical discolouration, and a massive presence of gum leaking from the cankers (Fig. 1a, b). One type of colony, characterized by fluffy, gray to dark gray, turning to-black mycelium, was consistently obtained from the isolations, and grouped as a potential *Botryosphaeriaceae* species, as suggested by Slippers and Wingfield (2007) (Fig. 1c). Conidia harvested within pycnidia, as well as from cirri, were hya- line, non-septate and ellipsoid, having a typical fusicoccum-like shape (Fig. 1d, e). Measurements resulted from this study are summarized in Table 1. Preliminary BLASTn searches of sequences derived from this study (GenBank Accession numbers: ITS MZ215772-76; EF-1α MZ229670-74; tub2 MZ229675-79) on both databases resulted in high identity scores with *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slip- pers & A.J.L. Phillips. MP analysis shows that our isolates clustered strongly with the type specimen of *N. parvum* (CMW9081) (bootstrap support 95) (Fig. 2). Tree scores of the heuristic search are summarized in Fig. 2. Pathogenicity tests showed that *N. parvum* is highly aggressive, being able to induce severe wood discolourations, bark cracking and a massive production of gum from the wounds on all the inoculated plants (Fig. 3a, b). The 3-years-old plants started to produce gum six days after the inoculation, and after 17 days two plants were completely dead. The lesion length average was 72 mm. Plants inoculated in the nursery began the production of gum three weeks after the inoculation. After ten weeks two plants were completely dead (Fig. 3c) and the lesion length average was 82 mm.

No lesions were detected on controls (Fig. 3d, e), and re-isolations fulfilled Koch's postulates. Results of our study confirm, for the first time, the new host association of *N. parvum* on the genus *Brachychiton*. Although few previous reports investigated Botry-osphaeriaceae attacking *B. acerifolius* and *B. populneus*, specifically *Diplodia* sp., *Dothiorella* sp., and *Lasiodiplodia theobromae* (Alfieri *et al.,* 1984; Cook & Dubé, 1989; Sandini & Ferrin, 1992), no other studies have been published in recent years. Among *Botry-osphaeriaceae*, *N. parvum* is considered one of the most damaging, being widespread around the world and being able to attack many different hosts (Phillips *et al.,* 2013; Sakalidis *et al.,* 2013; Slippers & Wingfield, 2007). Recent taxonomic updates revealed the accom-modation of other species (*N. algeriense*, *N. italicum* and *N. pandanicola*) as synonyms of *N. parvum* (*N. parvum* species complex) (Zhang *et al.,* 2021), expanding our knowledge for a proper diagnosis and risk assessment. In Sicily, *N. parvum* is reported on many crops, such as avocado (Guarnaccia *et al.,* 2016), mango (Ismail *et al.,* 2013), loquat (Giambra *et al.,* 2016), grapevine (Mondello *et al.,* 2013), walnut (Gusella *et al.,* 2021) and citrus (Bezerra *et al.,* 2021). Presence of *N. parvum*, as well as other *Botryosphaeriaceae* spp. in the nurseries can occur through latent infections of the propagation material, representing a serious risk for the success of the cultivation in the field (Giménez-Jaime *et al.,* 2006; Billones-Baaijens *et al.,* 2013). In addition, presence of susceptible contiguous hosts in nurseries easily guaran- tee the inoculum survival and dispersion over the years. From the same nursery investigated in the present study, we previously reported *N. parvum* attacking severely *Ficus carica* (Aiello *et al.,* 2020), and the ornamental species *Rhaphiolepis indica* (Gusella *et al.,* 2019).The current dispersal of diverse populations of *N. parvum* throughout the world is probably the result of the repeat introductions of agricultural and ornamental plant material (Sakalidis *et al.,* 2013), in conjunction with the host neutral behavior, common for many other *Botryosphaeriaceae* (Slippers & Wingfield, 2007), for which the

speciation seems to be mediated by other environment factors (Sakalidis *et al.,* 2013). In Sicily, the increase of tropical crops plantations in the recent years, and the consequent import of propagation mate- rial from other European Countries, seems to be a facilitated pathway of introduction of *Botryosphaeriaceae* in the territory (G. Polizzi, unpublished data). Urban environments, although less investigated than the agricultural ones, represent an important route of introduction and dissemination of these fungi (Lopes *et al.,* 2016). It is well known that stress factors like drought, injuries, unsuitable planting sites, play a key role in *Botryosphaeriaceae* disease expression (Mehl *et al.,* 2013; Slippers & Wingfield, 2007). Ornamental urban trees, as well as in some case the potted plants, can also be considered more exposed to stress factors such as high temperatures (Heat Island Effect), suboptimal growth conditions (little area for root expansion), and frequent root disturbance from utilities (Tubby & Webber, 2010). Therefore, since *Brachychiton* spp. are planted in Italy as ornamental trees in parks and streets, it is fundamental to highlight the susceptibility of this group of plants to *N. parvum*, as well as to other *Botryosphaeriaceae*. Furthermore, urban trees often grow in a non-native environment and *Botryosphaeriaceae*, known to be endophytes, occupy the endophytic niche left open, normally occupied, in a native environment, by endophytes horizontally acquired (Slippers & Wing- field, 2007). The spread of *Botryosphaeriaceae* in nurseries and in urban environments requires urgent, sensitive and efficient strategies of diagnosis, especially for latent infections. To our knowledge, this is the first report worldwide of *N. parvum* attacking *Brachychiton* spp.

Table 1. Conidial measurements of *Neofusicoccum parvum* isolates generated in this study and comparison with the ex-type

Species	Isolate	Conidial size (µm) (L × W)[1]	Reference
N. parvum	Type	(12-) 15–19 (-24) × 4–6	Slippers *et al.*, 2004
N. parvum	Di3A-BR1	(15.0-) 17.0 ± 1.4 (-21.1) × (5.2-) 6.1 ± 0.5 (-7.3)	This study
N. parvum	Di3A-BR2	(14.9-) 17.3 ± 1.4 (-20.7) × (5.2-) 6.2 ± 0.7 (-8.0)	This study
N. parvum	Di3A-BR8	(13.3-) 17.7 ± 1.9 (-21.0) × (5.2-) 6.4 ± 0.6 (-7.5)	This study

Figure 1. Symptoms and details of *Neofusicoccum parvum*: (a) gummosis from infected trunk; (b) internal wood discol- ouration; (c) colony of 7 days old grown on PDA; (d) hyaline conidia (scale bar = 50 µm); (e) cirri of conidia oozing from pycnidia on the bark surface (scale bar = 2 mm)

Figure 2. One of 100 equally most parsimonious trees generated from Maximum Parsimony analysis of the three-gene (ITS + EF-1α + *tub2*) combined dataset. Numbers are bootstrap values from 1,000 replicates. Bootstrap values lower than 70 are not shown. Bar indicates the number of nucleotide changes

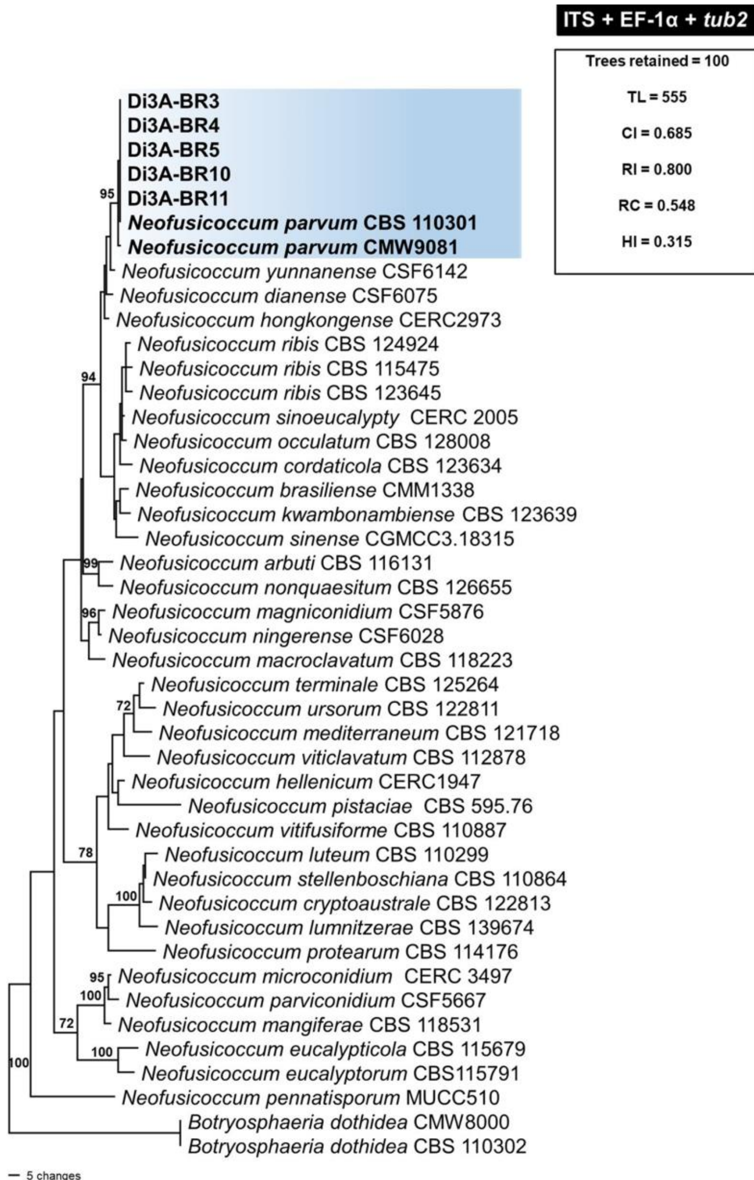
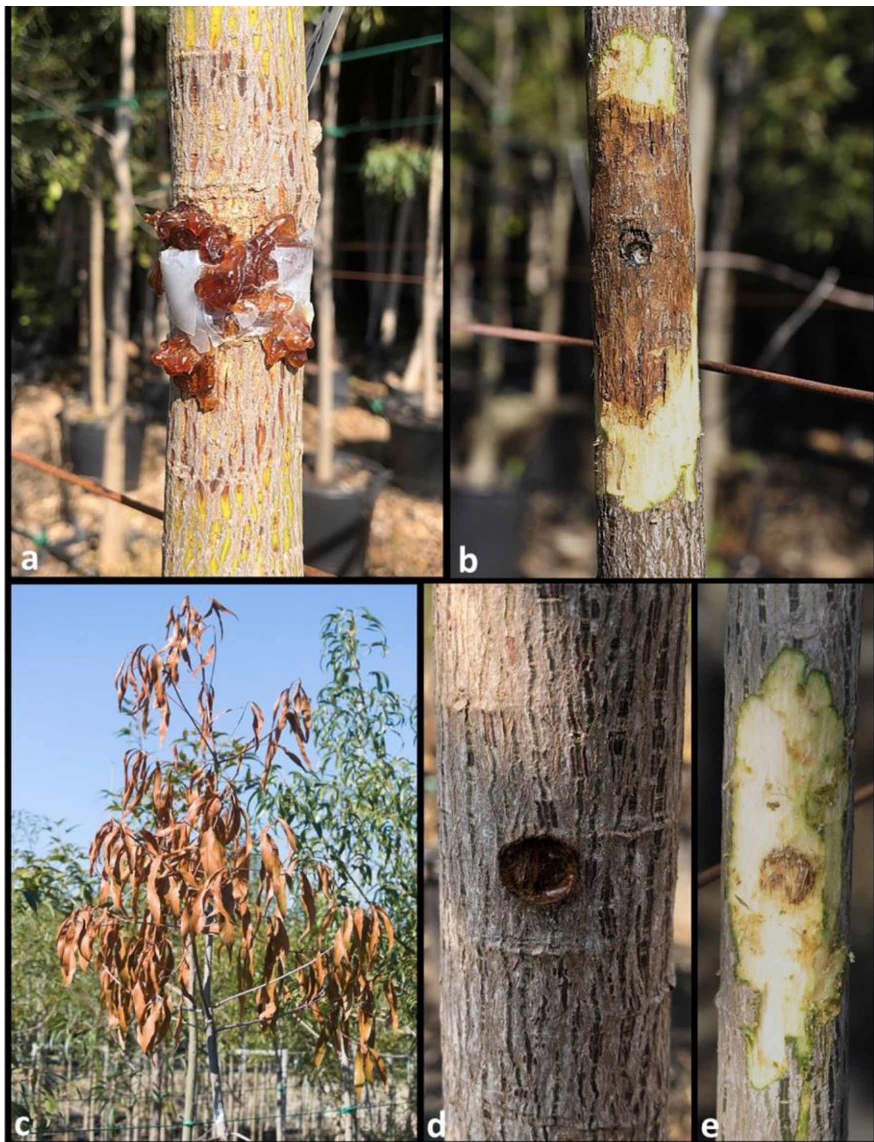


Figure 3. Pathogenicity test: (a) gummosis from the inoculation point and external canker progression; (b) internal wood discolouration after ten weeks; (c) dieback after ten weeks; (d, e) controls



8.2 References

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9. Other research activities: *Cylindrocladiella peruviana* and *Pleiocarpon algeriense* causing stem and crown rot on avocado (*Persea americana*)

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9.1 Introduction

Avocado (*Persea americana* Mill.) is native to Mexico but is spread around the world in tropical and subtropical regions. In southern Italy (Sicily), several farms of different extension coexist, directing their production to local market as well as European market (Migliore et al. 2018). Although the agronomic studies on this crop in Italy started more than 20 years ago, studies on phytopathological aspects have been limited. Recently, a wide study on branch cankers and stem-end rot conducted in the main avocado growing area in eastern Sicily revealed the presence of different species such as *Neofusicoccum parvum* (the highest virulent), *Diaporthe foeniculina* (=*D. foeniculaceae*), *D. sterilis*, *Colletotrichum gloeosporioides*, *C.*

fructicola, and a novel species *Neocosmospora perseae* (Guarnaccia et al. 2016, 2018). These reports show how woody cankers and stem-end rot could be considered the most important threats for avocado production in Italy so far. Avocado symptoms at the rhizosphere such as rot, discolouration, sunken lesions, and subsequent aspecific symptoms of the canopy such as wilt, leaves chlorosis/ browning, and decline, have been studied worldwide. These studies showed how several species within the Nectriaceae (Hypocreales, Ascomycetes) are involved in what is defined “Black root rot disease” (Dann et al. 2011, 2012; Parkinson et al. 2017a, b). A recent research conducted in Australia demonstrated that several *Dactylonectria* spp. and *C. ilicicola* are pathogenic to avocado, compared to other isolates of *Ilyonectria* sp., *Cylindrocladiella pseudoinfestans* and *Gliocladiopsis peggii* that did not result pathogenic (Parkinson et al. 2017b). Several reports also include the species *Ilyonectria destructans* (often with previous binomial denomination) associated with black root rot of avocado (Besoain and Piontelli 1999; Zilberstein et al. 2007; Ramírez-Gil and Morales-Osorio 2013) and *Ilyonectria macrodidyma* (Vitale et al. 2012). However, due to the numerous taxonomic changes and limited pathogenicity tests, *Ilyonectria* spp. were considered not pathogenic to this crop (Parkinson et al. 2017b), although species of this genus are well known as pathogen to many other plants (Úrbez-Torres et al. 2012; Lombard et al. 2013; Aiello et al. 2014, 2015). Among *Cylindrocladiella* species, *C. parva* has been found on roots and cuttings of avocado in South Africa (Crous et al. 1991) and associated with the dead 3-year-old avocado plants in Australia (Dann et al. 2012) and *C. pseudoinfestans* was collected from symptomatic avocado trees cultivated in nursery although theirs pathogenicity was not demonstrated (Parkinson et al. 2017b). *C. peruviana*, has been previously reported associated with root decay and cutting rot symptoms, but no data are available about its pathogenicity (Crous 2002; Van Coller et al. 2005). Root rot caused by the oomycete *Phytophthora cinnamomi*, and collar rot caused by *P. citricola* have

been considered for decades the most important root diseases in avocado orchards (Erwin and Ribeiro 1996).

Recently in Italy, young avocado trees showing symptoms of stem and crown rot were observed and brought to our laboratory for further investigations. The aim of the present study is to identify the fungal species associated with those symptoms and to evaluate their ability to induce symptoms on seedlings and young trees of avocado.

9.2 Materials and methods

9.2.1 Field sampling, isolations and morphological characterization

During the autumn of 2018, 20 young 3-year-old trees of avocado cv "Hass" from Campobello di Mazara, Trapani province (Sicily, Italy) were sampled, and analyses have been conducted in the laboratory. All the samples showed symptoms of stem and crown rot. Occasionally, as a consequence of severe infection, root rot was also observed. Small sections of basal stem and crown tissue 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, Oxoid, Basingstoke, UK) amended with 100 mg/l of streptomycin sulfate (Sigma- Aldrich, USA) to prevent bacterial growth, and then incubated at 25 ± 1 °C for 5–7 days. Single-conidial cultures were transferred on synthetic nutrient-poor agar (SNA; Nirenburg 1981) for morphological characteriza- tion. The morphological characteristics were determined with 30 measurements at × 1000 magnification of co- nidia mounted in lactic acid.

9.2.2 Molecular characterization and phylogenetic analysis

Genomic DNA was extracted from fourteen isolates (Table 1) using

the Wizard Genomic DNA Purification Kit (Promega Corporation, WI, USA). Species identifi- cation was achieved through DNA amplification and sequencing of a combined data set of loci: the nuclear ribosomal internal transcribed spacer (ITS) region, par- tial regions of the β-tubulin (*tub2*), the translation elon- gation factor-1α (*tef1*), the histone H3 (*his3*) and the RNA polymerase II second largest subunit (*rpb2*) genes. The primers used were ITS5 and ITS4 (White et al. 1990), T1 and Bt-2b (O'Donnell and Cigelnik 1997), EF1-728F and EF1-986R (Carbone and Kohn 1999), CYLH3F and CYLH3R (Crous et al. 2004), RPB2-5F2 and RPB2-7cR (O'Donnell et al. 2007), respectively. The PCR amplification mixtures and cycling conditions were adopted for the two loci were followed as described by Guarnaccia and Crous (2018) and Aigoun-Mouhous et al. (2019). Both strands of the PCR products were sequenced by Eurofins Genomics Service (Ebersberg, Germany). The generated DNA sequences were analyzed and consensus sequences were computed using the Geneious v. 11.1.5 software (Auck- land, New Zealand).
Novel sequences obtained in this study were blasted using the NCBIs GenBank nucleotide database, to iden- tify the most similar relatives for a taxonomic frame- work of the studied isolates. Alignments of different gene regions, including sequences obtained from this study and those downloaded from GenBank, were ini- tially performed by using the MAFFT v. 7 online server (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley 2013), and then manually adjusted in MEGA v. 7 (Kumar et al. 2016).

Phylogenetic analyses were conducted for establish- ing the identity of the isolates at species level, first individually for each locus (data not shown) and then as combined analyses of five loci. Additional reference sequences were selected based on recent literature of Nectriaceae species (Polizzi et al. 2012, Aiello et al. 2014, Lombard et al. 2015, Aigoun-Mouhous et al. 2019, Marin-Felix et al. 2019a, b). Phylogenetic analy- ses were based on Maximum

Parsimony (MP) for the individual loci and on MP and Bayesian Inference (BI) for the multi-locus analyses. Related with BI, the best evolutionary model for each partition was determined using MrModeltest v. 2.3 (Nylander 2004) and incorpo- rated into the analysis. MrBayes v. 3.2.5 (Ronquist et al. 2012) was used to generate phylogenetic trees under optimal criteria per partition. The Markov Chain Monte Carlo (MCMC) analysis used four chains and started from a random tree topology. The heating parameter was set to 0.2, and trees were sampled every 1000 generations. Analyses stopped at the moment which the average standard deviation of split frequencies was below 0.01. The MP analyses were conducted using PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10; Swofford 2003). Phylogenetic relationships were estimated by heuristic searches with 100 random addition sequences. Tree bisection-reconnection was used, with the branch swapping option set on 'best trees' only with all characters weighted equally and alignment gaps treated as missing data. Tree length (TL), consis- tency index (CI), retention index (RI) and rescaled con- sistence index (RC) were calculated for parsimony and bootstrap analyses (Hillis and Bull 1993), which were based on 1000 replications. Sequences generated in this study are deposited in GenBank (Table 1).

9.2.3 Pathogenicity and virulence on seedlings of avocado

Pathogenicity assays with two fungal species (*Pleiocarpon algeriense* and *Cylindrocladiella peruviana*) isolated from the avocado samples were per- formed to fulfil Koch's postulates. Simultaneously, the decay amounts (symptom severities on basal stem and crown as measure of relative virulence) associated to each fungal isolate were evaluated and compared using young seedlings and avocado plants. Four type isolates were selected from the sample subset of isolates comprising two strains of *Pleiocarpon algeriense*, Di3A-AP26 and Di3A-AP50, and two isolates of *Cylindrocladiella peruviana,* Di3A-

AP41 and Di3A-AP39. Pathogenicity of these isolates was assayed on healthy, 5-months-old seedlings of avocado. In detail, 12 inoculated plants for each fungal isolate were arranged in a randomized complete block design (RCBD) with three replicates, each consisting of four inoculated seedlings. To this aim, a piece of bark was removed at the base of the stem with a 6 mm diameter cork-borer and 6-mm-diameter mycelial plugs taken from a 12-day-old fungal colony were ap- plied as previously reported (Aiello et al. 2014, 2015, 2017). The wounds were covered with Parafilm to pre- vent desiccation. Controls consisted of sterile PDA plugs applied similarly to healthy young 12 seedlings. All avocado plants were kept in a growth chamber, with a 12 h photoperiod at 25 °C ± 1 °C and regularly watered. Disease incidence (DI, percentage of symptomatic plants), symptom severity (as lesion length - SSL) of the resulting lesions was recorded 2 months after inoculation. Re-isolations were performed for seedlings to confirm their pathogenicity. The experiments were per- formed twice.

9.2.4 Pathogenicity and virulence on young trees of avocado

Two isolates (Di3A-AP50 and Di3A-AP39) were inoculated onto 18 healthy 3-years-old avocado trees according to a randomized complete block design with three replicates, each consisting of three young trees. In detail, a piece of bark was removed at the base of the stem using a 7.5 mm diameter cork-borer and a mycelial plug of equal size taken from a 12-day-old fungal colony was applied. Controls consisted of sterile PDA plugs applied to nine healthy young trees. All avocado plants were kept in a growth chamber, with a 12 h photoperiod at 25 °C ± 1 °C and regularly watered. Disease incidence (DI, percentage of symptomatic plants) and symptom severity (as lesion length - SSL, and lesion depth - SSD expressed in mm) of the resulting lesions were recorded four months after the inoculations. Re-isolations were performed from artificially infected avocado trees to confirm their pathogenicity. The experiments were performed twice.

9.2.5 Data analysis

Data concerning virulence of *Pleiocarpon algeriense* and *Cylindrocladiella peruviana* isolates on avocado plants cv 'Hass' from the repeated experiment were analysed by using the Statistica package software (version 10; Statsoft Inc., Tulsa, OK, USA). The arithmetic means of DI and SS values [expressed both as lesion length (= SS_L) and as lesion depth (= SS_D)] were calculated averaging the values determined for each replicates of each treatment. Percentage DI data were transformed into the arcsine ($\sin^{-1}$ square root x) prior to analysis of variance (ANOVA), whereas both SS values were not transformed. Initial analyses of DI and SS data were performed by calculating F and *P* values associated to evaluate whether the effects of single factor (fungal isolate) and isolate × trial interactions are significant on single disease parameters. In the post-hoc analyses, the corresponding mean values of DI and SS were subsequently separated by the Fisher's least significance difference test (α = 0.05). Untransformed arithmetic means of DI and SS are presented in the Tables 2 and 3.

9.3 Results

9.3.1 Field sampling, isolation and morphological characterization

A widespread wilting of avocado plants was observed in one orchard located in Campobello di Mazara (Trapani province) where approximately 2,200 plants were cultivated. Disease incidence was approximately 40%. Symptomatic plants showed symptoms of stem and crown rot and subsequent stunted growth, general wilting and leaf chlorosis. Moreover, basal stem and crown were dry, cracked and showed external decay and internal discolouration of tissues, which sometimes resulted in the detachment of the bark and/or of roots from the stem (Fig. 1). Occasionally, in an advanced stage of infection root

rot was also observed. More of 800 plants were dead. Nectriaceae-like fungi colonies were consistently obtained from isolation of symptomatic tissues obtained from 20 plants and divided in two typologies depending on their appearance in culture. A total of 34 isolates were obtained from a single conidium or hyphal tip of pure cultures on PDA at 25 °C ± 1 °C. Based on the microscopic observations, the isolates of *Cylindrocladiella peruviana* produced conidia cylindrical, rounded at both ends, straight, 1-septate, 8 to 13 × 2 to 3 µm in accordance with the description by Agustí- Brisach et al. (2012). *Pleiocarpon algeriense* produced microconidia and macroconidia straight to curved, 1–5- septate, predominantly 3, 4-septate. Macroconidia 3-septate were 41 to 48 × 5 to 7 µm while conidia 4- septate measured 52 to 64 × 7 to 8 µm in accordance with the description by Aigoun-Mouhous et al. (2019). All the isolates were stored in the collection (label name Di3A-AP) of Dipartimento di Agricoltura, Alimentazione e Ambiente (Di3A), Catania, Italy. Moreover, two isolates of each typology were stored in the collection of Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

9.3.2 Molecular characterization and phylogenetic analysis

Six alignments were analysed representing single gene analyses of ITS, *tef1*, *tub2*, *his* and *rpb2*, and a combined alignment of the five genomic loci. The alignments pro- vided topologically similar trees. The combined species phylogeny consisted of 65 sequences, including the sequences of *Xenogliocladiopsis cypellocarpa* (culture CBS 133814) as outgroup. A total of 3193 characters (ITS: 1–538, *tef1*: 545–1060, *tub2*: 1067–1782, *his*: 1789–2333, *rpb2*: 2340–3193) were included in the phylogenetic analysis; 1245 characters were parsimony-in- formative, 383 were variable and parsimony-uninformative, and 1541 were constant. A maximum of 1000 equally most parsimonious trees were saved (Tree length = 5390, CI = 0.559, RI = 0.836 and RC = 0.467). Bootstrap support values from the

parsimony analysis are plotted on the Bayesian phylogenies in Fig. 2. For the Bayesian analyses, MrModeltest suggested that both the partitions should be analysed with dirichlet state frequency distributions. The following models were recommend- ed by MrModeltest and used: GTR + I + G for ITS, *tef1* and *his*, HKY + I + G for *tub2*, SYM + G for *rpb2*. In the Bayesian analysis, the ITS *tef1*, *tub2*, *his* and *rpb2* partitions had 210, 377, 443, 279 and 350 unique site patterns, respectively. The analysis ran for 4,770,000 generations, resulting in 4771 trees of which 3579 were used to calculate the posterior probabilities. In the combined analysis, eight representative isolates from avocado roots clustered with the ex-type of *P. algeriense*. A further six isolates identified as *C. peruviana*, formed with two reference strains, a highly supported subclade (1.00/100) in the broad group of *Cylindrocladiella* spp. The individual alignments and trees of the five single loci used in the analyses were compared with respect to their performance in species recognition.

9.3.3 Pathogenicity and virulence on avocado

In *in vivo* experiments (both on seedlings and trees of avocado) a significant effect of fungal isolate was al- ways detected on SS parameters (*P* value<0.0001) except for DI values since they were always 100%. Since isolate × trial interactions were always not signif- icant (P value >0.6) for all detected SS parameters (Tables 2 and 3) the two trials regarding both avocado seedlings and young trees were combined and statistically analysed. Pathogenicity and virulence on seedlings of avocado 2 months after artificial inoculation of both fungi, symptoms produced in seedlings were similar to those present in the orchard. These consisted of stem and crown rot. In correspondence to the inoculated site, above the bark was visible necrotic lesion which also extended under the bark (Fig. 3). No symptoms were observed on control plants. The pathogens were re-isolated from symptomatic tissues, thus fulfilling Koch’s postulates.

Regarding disease severities in the *post-hoc* analyses, the tested fungal isolates revealed a different pathogenic behaviour, resulting *P. algeriense* species more virulent than *C. peruviana* species. Moreover, *P. algeriense* Di3A-AP50 resulted the most aggressive isolate in inducing symptoms on avocado seedlings among all test- ed isolates (Table 2).
Four months after artificial inoculation of both fungi, symptoms produced in trees were similar to those present in the orchard. These consisted of necrotic lesions above the bark in correspondence to the inoculated site (Fig. 1). No symptoms were observed on control plants. The pathogens were re-isolated from symptomatic tis- sues, thus fulfilling Koch's postulates. As shown in Table 3, *P. algeriense* Di3A-AP50 caused always SS_L and SS_D values significantly higher than those recorded for *C. peruviana* Di3A-AP39 (Table 3).

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

Species	Culture no.[1]	GenBank no.[2]				
		ITS	*tef1*	*tub2*	*his*	*rpb2*
Calonectria ilicicola	CBS 190.50[T]	GQ280605	AY725726	AY725631	AY725676	KM232307
Cylindrocladiella addiensis	CBS 143794[T]	MH111383	MH111393	MH111388	–	–
Cy. australiensis	CBS 129567[T]	JN100624	JN099060	JN098747	JN098932	–
Cy. brevistipitata	CBS 142786[T]	–	MF444940	MF444926	–	–
Cy. camelliae	IMI 346845	AF220952	JN099087	AY793471	AY793509	KM232304
Cy. clavata	CBS 129564[T]	JN099095	JN098974	JN098752	JN098858	–
Cy. cymbiformis	CBS 129553[T]	JN099103	JN098988	JN098753	JN098866	–
Cy. elegans	CBS 338.92[T]	AY793444	JN099039	AY793474	AY793512	–
Cy. ellipsoidea	CBS 129573[T]	JN099094	JN098973	JN098757	JN098857	–
Cy. hawaiiensis	CBS 129569[T]	JN100621	JN099057	JN098761	JN098929	–
Cy. horticola	CBS 142784[T]	MF444911	MF444938	MF444924	–	–
Cy. humicola	CBS 142779[T]	MF444906	MF444933	MF444919	–	–
Cy. infestans	CBS 111795[T]	AF220955	JN099037	AF320190	AY793513	–
Cy. kurandica	CBS 129577[T]	JN100646	JN099083	JN098765	JN098953	–
Cy. lageniformis	CBS 340.92[T]	AF220959	JN099003	AY793481	AY793520	KM232303
Cy. lanceolata	CBS 129566[T]	JN099099	JN098978	JN098789	JN098862	–
Cy. lateralis	CBS 142788[T]	MF444914	MF444942	MF444928	–	–
Cy. longiphialidica	CBS 129557[T]	JN100585	JN098966	JN098790	JN098851	–

Cy. longistipitata	CBS 116075^{T}	AF220958	JN098993	AY793506	AY793546	–
Cy. microcylindrica	CBS 111794^{T}	AY793452	JN099041	AY793483	AY793523	–
Cy. natalensis	CBS 114943^{T}	JN100588	JN099016	JN098794	JN098895	–
Cy. nauliensis	CBS 143792^{T}	MH111387	MH111397	MH111392	–	–
Cy. nederlandica	CBS 152.91^{T}	JN100603	JN099033	JN098800	JN098910	–
Cy. novazelandica	CBS 486.77^{T}	AF220963	JN099050	AY793485	AY793525	–
Cy. parva	CBS 114524	AF220964	JN099009	AY793486	AY793526	
Cy. peruviana	CBS 114953	JN099123	JN099006	JN098805	JN098885	–
	IMUR 1843^{T}	AF220966	JN098968	AY793500	AY793540	–
	Di3A-AP39	*MT613323*	*MT510671*	*MT510677*	*MT635005*	*MT634991*
	Di3A-AP41	*MT613324*	*MT510672*	*MT510678*	*MT635006*	*MT634992*
	Di3A-AP44	*MT613325*	*MT510673*	*MT510679*	*MT635007*	*MT634993*
	Di3A-AP45	*MT613326*	*MT510674*	*MT510680*	*MT635008*	*MT634994*
	Di3A-AP47	*MT613327*	*MT510675*	*MT510681*	*MT635009*	*MT634995*
	Di3A-AP48	*MT613328*	*MT510676*	*MT510682*	*MT635010*	*MT634996*
Cy. pseudocamelliae	CBS 129555^{T}	JN100577	JN098958	JN098814	JN098843	–
Cy. pseudohawaiiensis	CBS 210.94^{T}	JN099128	JN099012	JN098819	JN098890	–
Cy. pseudoinfestans	CBS 114531^{T}	AF220957	JN099004	AY793508	AY793548	–
Cy. pseudoparva	CBS129560^{T}	JN100620	JN099056	JN098824	JN098927	–
Cy. queenslandica	CBS 129574^{T}	JN099098	JN098977	JN098826	JN098861	–
Cy. reginae	CBS 142782^{T}	MF444909	MF444936	MF444922	–	–
Cy. stellenboschensis	CBS 110668^{T}	JN100615	JN099051	JN098829	JN098922	–
Cy. terrestris	CBS 142789^{T}	MF444915	MF444943	MF444929	–	–

Cy. thailandica	CBS 129571[T]	JN100582	JN098963	JN098834	JN098848	–
Cy. variabilis	CBS 129561[T]	JN100643	JN099080	JN098719	JN098950	–

Table 1 (continued)

Species	Culture no.[1]	GenBank no.[2]				
		ITS	*tef1*	*tub2*	*his*	*rpb2*
Cy. viticola	CBS 112897[T]	AY793468	JN099064	AY793504	AY793544	–
Cy. vitis	CBS 142517[T]	KY979751	KY979891	KY979918	–	–
Dactylonectria macrodidyma	CBS 112615[T]	AY677290	JF268750	AY677233	JF735647	JF268710
D. torresensis	CBS 129086[T]	JF735362	JF735870	JF735492	JF735681	KM232347
Ilyonectria capensis	CBS 132815	JX231151	JX231119	JX231103	JX231135	KM232336
I. palmarum	CBS 135753	HF937432	HF922615	HF922609	HF922621	–
Neonectria ditissima	CBS 100316	KM515890	KM515944	DQ789858	–	DQ789787
Pleiocarpon algeriense	CBS 144964[T]	MH587320	MH587323	MH587324	MH587296	MH587322
	Di3A-AP26	*MT613330*	*MT597130*	*MT597138*	*MT635011*	*MT634997*
	Di3A-AP27	*MT613331*	*MT597131*	*MT597139*	*MT635012*	*MT634998*
	Di3A-AP28	*MT613332*	*MT597132*	*MT597140*	*MT635013*	*MT634999*
	Di3A-AP29	*MT613333*	*MT597133*	*MT597141*	*MT635014*	*MT635000*
	Di3A-AP31	*MT613334*	*MT597134*	*MT597142*	*MT635015*	*MT635001*
	Di3A-AP50	*MT613335*	*MT597135*	*MT597143*	*MT635016*	*MT635002*
	Di3A-AP51	*MT613336*	*MT597136*	*MT597144*	*MT635017*	*MT635003*

	Di3A-AP52	*MT613337*	*MT597137*	*MT597145*	*MT635018*	*MT635004*
P. livistonae	CBS 145030^{T}	MK539963	MK540165	MK540179	MK540234	MK540095
P. strelitziae	CBS 142251^{T}	KY304644	KY304722	KY304750	KY304616	KY304697
	CBS 142252	KY304663	*KY304741*	*KY304769*	KY304635	KY304713
	CPC 27629	KY304645	*KY304723*	*KY304751*	KY304617	KY304698
Thyronectria quercicola	CBS 128976^{T}	JF832624	JF832581	JF832880	KM231595	KM232411
Xenogliocladiopsis cypellocarpa	CBS 133814^{T}	KM231760	KM231885	KM232017	KM231479	KM232332

[1] CPC: Culture collection of P.W. Crous, housed at Westerdijk Fungal Biodiversity Institute; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; Di3A: Dipartimento di Agricoltura, Alimentazione e Ambiente, Catania, Italy. IMI: International Mycological Institute, CABI–Bioscence, Egham, Bakeham Lane, U.K.; IMUR: Institute of Mycology, University of Recife, Recife, Brazil. Ex–type and ex–epitype cultures are indicated with ^{T}

[2] ITS: the nuclear ribosomal internal transcribed spacer region; *tef1*: partial translation elongation factor 1–α gene; *tub2*: partial beta–tubulin gene; *his*: the histone H3 gene; *rpb2*: the RNA polymerase II second largest subunit gene. Sequences generated in this study are indicated in italics

Table 2. Pathogenicity and compared virulence among Pleiocarpon algeriense and Cylindrocladiella peruviana isolates on avocado seedlings.

Isolate	DI (%)[x]	SS (lesion length - mm)[x]
Pleiocarpon algeriense Di3A-AP50	100	35.44 ± 1.66 a
Pleiocarpon algeriense Di3-AP26	100	18.33 ± 0.36 b
Cylindrocladiella peruviana Di3A-AP41	100	12.18 ± 0.69 c
Cylindrocladiella peruviana Di3A-AP39	100	13.3 ± 0.99 c
	–	F (isolate) = 224.945; P = 0.000[z] F (isol. × trial) = 0.012; P = 0.998[ns]

[x] Data derived from two repeated experiments. Means are from three replicates (each consisting of four plants). ± standard error of the mean (SEM); Values followed by the same letters within the column are not significantly different according to the Fisher's least significance differences test (α = 0.05)

[z] F test and associated P value of fixed effects; ns = not significant

Table 3. Pathogenicity and compared virulence between *Pleiocarpon algeriense* and *Cylindrocladiella peruviana* isolates on avocado young trees.

Isolate	DI (%) [x]	SS_L (lesion length, mm) [x]	SS_D (lesion depth, mm) [x]
Pleiocarpon algeriense Di3A-AP50	100	83.67 ± 1.6 a	1.92 ± 0.11 a
Cylindrocladiella peruviana Di3A-AP39	100	15.67 ± 0.09 b	1.0 ± 0.00 b
	–	*F (isolate) = 3435.4; P = 0.000* [z] F (isol×trial) = 2.05; *P* = 0.19 [ns]	F (isolate) = *44.73; P = 0.000* [z] F (isol×trial) = 0.24; *P* = 0.64 [ns]

[x] Data derived from two repeated experiments. Means are from three replicates (each consisting of three plants). ± standard error of the mean (SEM); Values followed by the same letters within the column are not significantly different according to the Fisher's least significance differences test ($\alpha = 0.05$)

[z] F test and associated *P* value of fixed effects; ns = not significan

Figure 1. Symptoms caused by Cylindrocladiella peruviana and Pleiocarpon algeriense on avocado plants. a stunted growth, wilting and leaf chlorosis; b, c, d stem and crown rot; e, f symptoms on artificially inoculated young trees with *Cylindrocladiella peruviana* (e) and *Pleiocarpon algeriense* (f).

Figure 2. Consensus phylogram of 4771 trees resulting from a Bayesian analysis of the combined ITS, tef1, tub2, his and rpb2 sequences. Bayesian posterior probability and bootstrap support values are indicated at the nodes. Bold indicates ex–type strains. The tree was rooted to *Xenogliocladiopsis cypellocarpa* (CBS 133814)

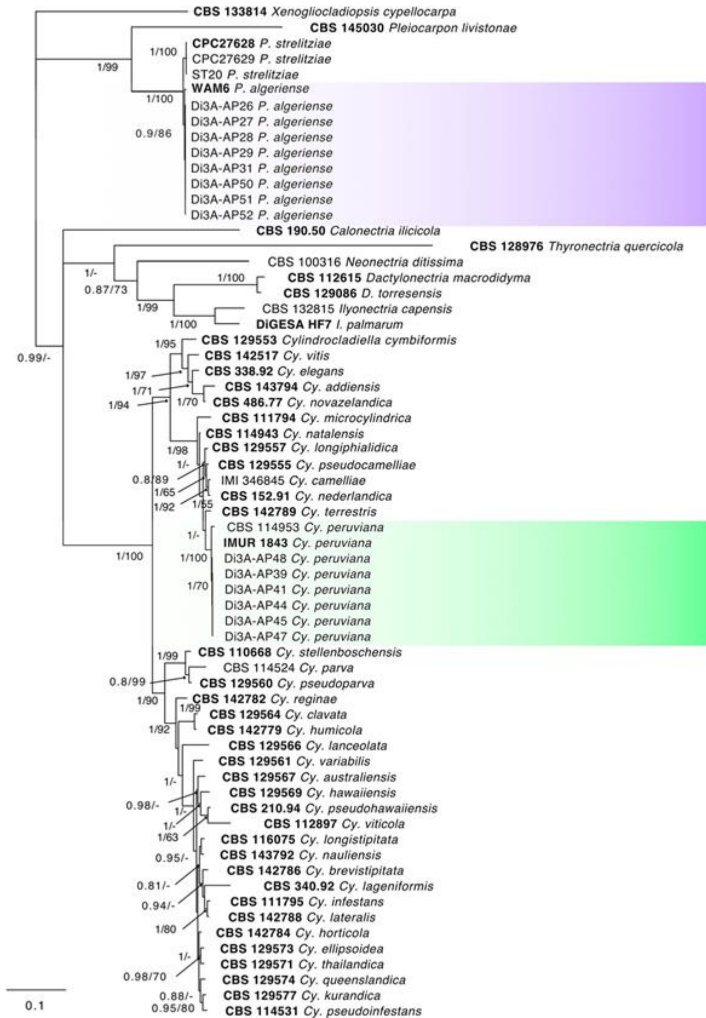

Figure 3. Symptoms on artificially inoculated seedlings with Pleiocarpon algeriense and Cylindrocladiella peruviana. Internal necrotic lesions caused by Pleiocarpon algeriense (a, b) and internal necrotic lesions and stem and crown rot caused by *Cylindrocladiella peruviana* (c, d).

9.4 Discussion

This study reveals for the first time the presence and pathogenicity of *C. peruviana* and *P. algeriense* on avocado. The polyphasic approach based on morpho- logical and molecular analyses permitted to distinguish the collected strains at the species level within the broad genera of *Cylindocladiella* and *Pleiocarpon*.

Cylindrocladiella (Hypocreales, Nectriaceae) ge- nus includes soil-borne fungi, often reported in sever- al studies with the name of *Cylindrocladium,* lately separated in two distinct genera, having *Calonectria* and *Nectricladiella* teleomorphs respectively (Boesewinkel 1982; Crous and Wingfield 1993; Crous et al. 1994; Schoch et al. 2000; Crous 2002). *Cylindrocladiella* spp. are frequently associated with root rot diseases of many plant species, e.g. on woody plants like *Eucalyptus* spp. and *Pinus* sp. (Boesewinkel 1986; Mohanan and Sharma 1985; Lombard et al. 2012). In Sicily, several studies have been conducted on crown and root rot of various plant hosts, revealing how species of the close genus *Cylindrocladium* (*Calonectria*) are widely spread across different hosts (Polizzi et al. 2007, 2012; Vitale et al. 2009, 2013). Different species of *Cylindrocladiella* including *Cy. peruviana* have been found on roots and cuttings of avocado. However, theirs pathogenicity has not been demonstrated on this crop (Darvas 1978; Crous et al. 1991; Van Coller et al. 2005; Dann et al. 2012; Parkinson et al. 2017b). Agustí-Brisach et al. (2012) reported *Cy. peruviana* for the first time in Spain associated with black-foot disease of grapevine, referring symptoms of reduced vigor, necrotic root lesions, and occasionally mortality. Similarly, Álvarez et al. (2012) in Perù and Koike et al. (2016) in California reported *Cy. peruviana* and other Nectriaceae involved in black- foot disease of grapevine. These recent reports, including the present study, confirm that this fungus plays a role in causing diseases, although *Cylindrocladiella* spp. are not typically considered primary pathogen s (Lombard et al. 20 12 ; Jayawardena et al. 2018).

Regarding the other nectriaceous species reported in this study, Aiello et al. (2017) described the new genus *Pleiocarpon* and *P. strelitziae* sp. nov. in Italy causing basal stem and root rot of the ornamental *Strelitzia reginae.* Pathogenicity tests demonstrated the aggres- sive pathogenic nature of *P. strelitziae*, resulting in mortality of all inoculated test plants within 2 months (Aiello et al. 2017). Recently, two new species of *Pleiocarpon* have been described; in particular *P. livistonae* from *Livistona rotundifolia* (Arecaceae) in Sri Lanka, causing root and corm rot (Marin-Felix et al. 2019a), and *P. algeriense* isolated in Algeria from grapevine cuttings with black-foot disease (Aigoun- Mouhous et al. 2019). Results of this studies demon- strated the pathogenicity of *C. peruviana* and *P. algeriense* on avocado. Moreover, the tested fungal isolates revealed a different pathogenic behaviour, resulting *P. algeriense* species more virulent than *C. peruviana* species. However, the symptoms observed in orchard could be the result of the interactions between different Nectriaceae species with different virulence levels as reported by some studies (Tewoldemedhin et al. 2011; Whitelaw-Weckert et al. 2013). Further evidences will be required to investigate the role of the single species involved in the disease and their interactions in pathogenesis. Prevention is the first strategy to manage these diseases. Affected plants produced in nursery could represent the primary way for nectriaceous spread through commercial orchards. Thus, the use of healthy plants and rapid fungi detection are crucial steps in prevention of stem and crown rot disease (Dann et al. 2012).

This study reports the presence and pathogenicity of two nectriaceous species on avocado in Italy for the first time, thereby the high risk of stem and crown rot in avocado commercial orchards. As described in this paper, *Cy. peruviana* and *P. algeriense* isolates recovered from infected stem and crown tissue, were able to cause different symptom severity levels among them. Further studies should be addressed to establish the most effective strategies to prevent their spread from the nursery to the open field.

9.5 References

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10. Conclusions

This PhD thesis is the result of three years of investigation in the field of Plant Pathology, focused on mycology; a cooperative effort between the University of Catania-Dept. of Agricoltura, Alimentazione e Ambiente, and the University of Davis- Kearney Agricultural Research and Extension Center.

During the last years, pistachio orchards are spreading in Sicily and new advanced forms of cultivation are increasing in the island, leading this crop to become more competitive and more productive. Although the Sicilian production cannot compete with the international demand of nuts, it represents an important opportunity for the Italian agricultural system, in terms of economic income, social development and national and international marketing visibility.

Considering the interest of growers, consumers, and public and private companies and institutions in promoting pistachio cultivation, it was crucial to deeply investigate the phytopathological situation, being one of the main limiting factor to the industry.

As attested in the introduction section, the aim of the thesis was to update our knowledge on fungal diseases affecting this crop. The state of art was very outdated and poorly investigated until the last recent years when our Department of Agricoltura, Alimentazione e Ambiente, started to conduct field surveys in Bronte (Catania province).

First results were obtained identifying new diseases and new pathogens previously unreported or undescribed, i.e., *Leptosillia pistaciae* (ex *Liberomyces pistaciae*) and *Arthrinium xenocordella*.

In my research activities we decided to expand the area of investigation including the new orchards in Agrigento and Caltanissetta provinces, that represent the most advanced reality of pistachio production in the island. We found that many orchards were attacked, with different disease incidence levels, by

Botryosphaeriaceae spp.

Specifically, we identified three species: *Botryosphaeria dothidea*, *Neofusicoccum hellenicum*, and *N. mediterraneum*. Our results showed new aspect of the etiology of this complex disease for pistachio in Italy, in accordance with other international scientific research, reporting also the species *N. hellenicum* for the first time in Italy.

At the same time, a very common and widespread disease of pistachio named "Leaf Spot", present in the entire territory and in all productive areas of the island and worldwide, was further investigated since no information on the causal agent, its biology, and its taxonomy were available since the first description of the disease in 1934.

Our new molecular data and phylogentic analysis showed that *Septoria pistaciarum* is the causal agent in Sicily of the Leaf Spot disease.

These results on pistachio fungal diseases opened an interesting new line of research, with the main goal to improve the quality and the yield of this crop not only in Sicily but in the Mediterranean area.

As described in the other chapters of the thesis, a conspicuous number of symptomatic samples belonging to tree crops and ornamental species, showing symptoms of *Botryosphaeriaceae* diseases, have been brought to our laboratory for further analysis.

All the phytopathological cases have been deeply investigated, revealing how complex are *Botryosphaeriaceae* diseases, and how relevant is their impact in the agricultural system and urban landscapes.

Due to the polyphagous nature of many species of this family, the adaptation to diverse environmental conditions and their diffusion in terms of epidemiology, it is crucial to do not underestimate *Botryosphaeriaceae* and their evolution.

For these reasons, the findings in this thesis have made a significant contribution to expanding scientific and industry knowledge.