UNIVERSITÀ
DI TORINO

Università degli Studi di Torino

PhD Programme in Biological Sciences
and Applied Biotechnologies

Molecular Characterization and Pathogenicity of Emerging Fungal Pathogens of Fruit and Leafy Vegetables

Tutor:
Davide Carmelo Spadaro

Co-tutor:
Vladimiro Guarnaccia

Candidate:
Muhammad Waqas

XXXV Cycle: 2019 /2024

Table of Contents

Abbreviations

A

AFLP= Amplified fragment length polymorphism

Alt-a1= Alternaria allergen a1

ATPase= adenosine triphosphatase

C

cmda= calmodulin

cv.= cultivar

cm= centimeter

D

DNA= Deoxyribonucleic Acid

E

endoPG= endo-polygalacturonase

F

FAO= Food and Agriculture Organization

f. sp.= *forma specialis*

FOL= *Fusarium oxysporum* f. sp. *lactucae*

G

GAPDH= Glyceraldehyde 3-phosphate dehydrogenase

H

HIS= histone

I

IGS= Intergenic spacer

ITS= Internal Transcribed Spacer

M

MLST= multi-locus sequence typing

mtLSU= mitochondrial large subunit

mtSSU= Mitochondrial small subunit

P

PDA= Potato Dextrose Agar

PCA= Potato Carrot Agar

PCR= polymerase chain reaction

R

RAPD= Random Amplified Polymorphic DNA

rpb2= RNA polymerase II second largest subunit

S

SCAR= Sequence Characterized Amplified Region

Spp.= species (plural)

T

tef-1α= translation elongation factor 1-alpha

THNR = 1,3,8-trihydroxynaphthalene reductase

tub2= beta-tubulin 2

U

μm= micrometer

General Introduction to the thesis chapters

Chapter 1: An overview of emerging fungal pathogens of fruits and leafy vegetables and their taxonomy based on morphology and molecular tools.

Chapter 2: A monitoring of postharvest diseases was conducted in sweet cherry harvested from orchards located in Piedmont, Northern Italy, over three years, from 2020 to 2022. A total of 180 isolates were isolated from black rotted cherries. The aim was to identify and characterize a selection of forty isolates of *Alternaria* spp. based on morphology, pathogenicity, and combined analysis of *rpb2*, Alt-a1, endoPG and OPA10-2. Based on the concatenated session of four gene regions, thirty-three out of forty isolates were identified as *A. arborescens* species complex (AASC), and seven as *A. alternata*. Pathogenicity was evaluated on healthy 'Regina' sweet cherry fruits. All the tested strains were pathogenic on their host. To the best of our knowledge this study represents the first report of AASC as an agent of black rot of sweet cherries in Italy.

Chapter 3: *Diaporthe* is one of the most widespread and cosmopolitan endophytic fungi. The aims of the chapter were, to isolate and identify the fungal species associated with hazelnut nuts defects in Northern Italy, to evaluate the genetic diversity of *Diaporthe* spp. associated with hazelnut nuts defects in Northern Italy, and to evaluate the pathogenicity of the species found. A total of 161 *Diaporthe* species were isolated from rotted hazelnuts, and 40 species of *Diaporthe* were selected for further analysis. Seven *Diaporthe* species (*D. eres*, *D. foeniculina*, *D. novem*, *D. oncostoma*, *D. ravennica*, *D. rudis*, and *D. sojae*) were identified based on morphological characterization and multi-locus phylogenetic analysis of the ITS, *tef1-* α, and *tub2*. Pathogenicity test performed on hazelnut nuts 'Tonda Gentile del Piemonte' using a mycelium plug showed that all the *Diaporthe* isolates were pathogenic on their original host. To our knowledge, this work is the first report of

D. novem, *D. oncostoma* and *D. ravennica* on hazelnut nuts worldwide. *Diaporthe foeniculina*, *D. rudis*, and *D. sojae* were reported for the first time as agents of hazelnut nut rot in Italy. In our study, *D. eres*, representing the main causal agents responsible for defects and nut rot of hazelnuts in Italy.

Chapter 4: This chapter represent the first report of nut rot caused by *Neofusicoccum parvum* on hazelnut (*Corylus avellana*) in Italy.

Chapter 5: Vascular wilt of lettuce is caused by strains of the *Fusarium oxysporum* species complex. According to their biological activity, these isolates are considered as members of the *forma specialis lactucae* (FOL). The aim of this chapter was to determine the race for some isolates of *F. oxysporum* f. sp. *lactucae*, and to establish the new species name according to Lombard et al., (2019) for the four races of *F. oxysporum* f. sp. *lactucae*, based on phylogenetic analyses. The race was determined through pathogenicity test with a set of differential lettuce cultivars for some isolates not previously characterized, which showed to belong to race 1 and race 4. Pathogenicity was performed on five species to confirm the Koch's postulates: lettuce cv. Romana Verde, lamb's lettuce cv. Trophy, spinach cv. Zebu, wild rocket cv. Winter and cultivated rocket cv. Coltivata. Pathogenicity tests showed that most isolates were pathogenic on lettuce with different levels of virulence; in particular, isolates of races 3 and 4 showed a higher level of virulence. Interestingly, isolates of FOL race 3 and 4 were pathogenic not only on lettuce but also on lamb's lettuce. Results of multigene phylogeny showed that *Fusarium* strains of race 1 and 4 belong to *F. curvatum*, strains of race 2 to *F. curvatum* and *F. odoratissimum* and strains of race 3 to *F. cugenangense*. Phylogenetically, race 4 is very close to race 1, but by considering the host spectrum, it shows some similarities to race 3.

Chapter 6: In this chapter identification, characterization (based on morphology and molecular tools), and pathogenicity of fungal pathogens are discussed. The

impact of identification and resolution of species has important benefits for epidemiology and the management of the disease.

Chapter 1

Introduction

Fruits and vegetables are an important source of nutrition for humans because they provide necessary growth factors such as minerals, fibers, vitamins, oil, and fats in a suitable proportion (Moura et al., 2022). The daily use of fruits and vegetables may reduce the risk of chronic diseases, including cardiovascular diseases, specific types of cancer, blood pressure, osteoporosis, chronic obstructive pulmonary diseases, hypercholesterolemia, respiratory problems, mental health as well as digestive problems (Wallace et al., 2020). In addition, the consumption of fruits and vegetables is associated with a reduction in mortality and risk of diabetes by preventing the weight gain (Olaya et al., 2019).

The annual production and consumption of fruits and vegetables is increasing due to the awareness of consumers towards their diet and health, and it also helps boost agricultural economic growth (Sivakumar and Bautista-Baños, 2014). China is the biggest producer and exporter of vegetables and fruits worldwide. Top ten fruit-producing countries are China, India, Brazil, Turkey, Mexico, Indonesia, the USA, Spain, Italy, and the Philippines. Whereas top ten vegetable-producing countries are China, India, the USA, Turkey, Vietnam, Nigeria, Egypt, Mexico, Russian Federation, and Spain (FAO, 2023).

Simultaneously, there are several challenges and threats to the production and processing of fruits and vegetables. These include pre-harvest factors (inadequate rainfall, changes in climatic conditions, pests, diseases, soil type, planting distances, irrigation, fertilization, and various other factors), post-harvest waste, and post-harvest losses in the field during storage, transportation, and commercialization from grower to consumers (El-Beltagi et al., 2023; Hasan et al., 2018). During post-harvest waste, fruits and vegetables are discarded because they do not meet the expectations of consumers for various reasons, such as taste, color, aroma, or

established preferences. On the other hand, post-harvest losses are described as incidental losses that may occur during storage, insect damage, internal bruising, physical damage, and premature spoilage (Porat et al., 2018; Shipman et al., 2021), reaching a significant amount of 25-50% that is third of the total amount of food produced worldwide (El-Beltagi et al., 2023).

Emerging fungal pathogens of fruit and leafy vegetables

Fruits and vegetables are perishable products (Pirozzi et al., 2021) with a high-water content, which makes them more vulnerable to fungal and bacterial diseases (Mostafa et al., 2021). However, fungi are considered the main cause of pre- and post-harvest diseases of fruits and vegetables such as *Alternaria* species, *Aspergillus* species, *Botrytis cinerea*, *Cladosporium*, *Colletotrichum*, *Diaporthe*, *Diplodia*, *Fusarium*, *Monilinia*, *Mucor*, *Phytophthora*, *Penicillium*, *Rhizopus*, *Sclerotinia*, and *Trichoderma* species (Khuna et al., 2022; Singh et al., 2018). Fungal infections may occur during the growing season (pre-harvest infection), harvest time, handling, storage, transportation, marketing chain, and consumption as post-harvest infection (Jianying et al., 2021). This thesis focuses on diseases of fruits and vegetables such as *Alternaria* rot of fruits caused by *Alternaria* spp., nut rot caused by *Diaporthe* spp., and *Neofusicoccum parvum*, and Fusarium wilt of lettuce caused by *Fusarium* spp., which are responsible for pre- and post-harvest decay of vegetables.

Alternaria rot of fruits

Alternaria fruit rot, also known as black rot (Timmer et al., 2003), is a prominent fungal disease in a wide variety of fruits, including apples, cherries, grapes, peaches, pears, plums, and pomegranate decay in the post-harvest stages (Ahmad et al., 2020; Cara et al., 2022; Szabó et al., 2023; Vitale et al., 2021). Alternaria rot contributes to the rapid deterioration of fruit quality and affects animal and human health through mycotoxin production (Miranda-Apodaca et al., 2023). It is responsible for

significant economic losses worldwide in favorable environmental conditions such as warm temperatures and high humidity and fruit losses may reach up to 50% (Manjunatha et al., 2022; Slathia et al., 2021). Alternaria rot is caused by members of the genus *Alternaria* (Wang et al., 2021) and was first identified by Nees in 1816 (Nees, 1816). The genus *Alternaria* belongs to Pleosporaceae family, Pleosporales order, Dothideomycetes class and Ascomycota phylum. It is ubiquitous fungal pathogen that includes pathogenic, endophytic, and saprobic species (Ramezani et al., 2019; Saharan et al., 2016). Currently, *Alternaria* genus is divided into twenty-nine sections with more than 360 species (He et al., 2024; Li et al., 2023). Several *Alternaria* spp. are responsible for the Alternaria rot of fruits from different fruit-growing regions worldwide (Singh et al., 2018). *Alternaria* spp., such as *Alternaria alternata*, *A. arborescens*, and *A. tenuissima*, are responsible to cause fruit rot of blueberries (Zhu and Xiao, 2015), rot of apples (Ntasiou et al., 2015), and fruit rot of Japanese plum (Riquelme et al., 2021). *A. botrytis*, *A. destruens*, and *A. rosae* have been previously reported in European pears (DeShields et al., 2021). *A. alternata* has been reported to cause rot of figs (Latinović et al., 2014), peaches (Abata et al., 2017), kiwifruits (Li et al., 2017), Opium Poppy (Guo et al., 2020), strawberries (Al-Rahbi et al., 2021), and cherries (Ahmad et al., 2020). *A. alternata* and *A. tenuissima* are associated with the black rot of sweet cherries (Cancino et al., 2023).

The symptoms of Alternaria rot vary depending on the fruit type and different stages of infection. However, common symptoms include the development of dark sunken lesions on the fruit surface. Most *Alternaria* spp. are weak pathogens; therefore, wounds or natural openings are needed for penetration into the host (Singh et al., 2018). Furthermore, the sugar content of the fruit increases, and fruit defense mechanisms are weakened, making fruits more susceptible to black rot. In later stages of the infection, under high humidity and temperature, the size of the lesions

increases gradually, and ultimately cover the complete fruit, as a result become soft (Xiang et al., 2021).

Diaporthe rot of fruit

Diaporthe rot is an economically important fungal disease of fruits that can infect a wide variety of fruits (Guarnaccia and Crous, 2017; Thomidis et al., 2019; Zabiák et al., 2023). It can cause considerable losses in the field or after harvest in terms of the quantity and quality of fruits, depending on the environmental conditions, fruit crop, and *Diaporthe* spp. virulence (Huang et al., 2013). Rotting is the main cause of fruit deterioration during storage (Zoffoli et al., 1998). *Diaporthe* fruit rot can cause losses up to 30% on peach and 80% on European pear (Bertetti et al., 2018; Zhang et al., 2021). Moreover, it can cause defects in hazelnut nuts and are responsible for reducing the quality of nuts (Battilani et al., 2018).

The *Diaporthe* genus belongs to the Diaporthaceae family, Diaporthales order, Sordariomycetes class and Ascomycota phylum (Ariyawansa et al., 2021; Dissanayake et al., 2017). *Diaporthe* is a cosmopolitan group of species that was originally introduced with *D. eres* as typified species recorded in Germany from *Ulmus* sp. (Nitschke, 1870). The members of the *Diaporthe* genus comprise endophytes, saprophytes, and fungal pathogens that are responsible for wide host range of fruit rot/nut rot diseases (Díaz et al., 2017; Guarnaccia et al., 2018; Thomidis et al., 2019; Xiao et al., 2022). Several species of *Diaporthe* have been associated with fruit rot, such as *D. eres*, which has been reported to cause fruit rot in Persimmon Fruits (Geng and Wang, 2023), walnut (Zabiák et al., 2023), and hazelnut defects (Battilani et al., 2018). *D. rudis* has been reported on European pears (Kc and Rasmussen, 2019) and grapes (Lorenzini and Zapparoli, 2019). *D. ambigua*, *D. australafricana*, *D. nobilis*, *D. novem*, and *D. passiflorae* are responsible for post-harvest rot of kiwifruit (Díaz et al., 2017; Li et al., 2019). *D. hongkongensis* and *D. fusicola* are associated with peach rot (Xiao et al., 2022;

Zhang et al., 2021). *D. foeniculina* has been reported to cause fruit rot in lemons (Tekiner et al., 2020). *D. sojae* causes fruit-brown rot disease in navel oranges (Xiao et al. 2023).

Fruit rot diseases caused by *Diaporthe* can show a variety of symptoms, some of which are specific to the host plant, such as circular, rotten, soft sunken lesions, and the appearance of pycnidia on the necrotic surface of lesions (Díaz et al., 2017; Xiao et al., 2022). Moreover, disease causes early defoliation of plants, fruit that fail to mature, remains undersized, and develops necrotic spots that turn into complete fruit rot when fully grown. The disease progresses rapidly and can lead to the complete loss of the crop, leaving growers with no marketable product. The disease has caused up to 100% loss in affected nurseries, but its prevalence each year is unpredictable, and there is limited knowledge about the factors that influence disease levels, such as environmental or host-related factors (Emanuel et al., 2023).

Fusarium oxysporum affecting vegetables

Fusarium oxysporum Schlecht is an economically important and commonly encountered species belonging to the family Nectriaceae, order Hypocreales, and phylum Ascomycota (Bahadur, 2021; Gordon, 2017). *Fusarium* spp. are ubiquitous soil-borne pathogens of a wide range of horticultural crops, such as asparagus, basil, beans, brassicas, coriander, cucurbits, lettuce, leek, onion, peas, peppers, spinach, and tomatoes (Bodah, 2017; Edel-Hermann and Lecomte, 2019).

Fusarium oxysporum includes different *formae speciales* (ff. spp.) based on their host specificity (Leslie and Summerell, 2006), and more than 150 plant species have been identified as susceptible hosts (Rana et al., 2017). The *forma specialis* concept is useful in identifying a group of isolates with an overlapping host range while avoiding the assumptions of homogeneity (Leslie, 2012). These isolates may have either a monophyletic or polyphyletic origin. Understanding the origin of these isolates is vital for developing effective disease control strategies, particularly when

considering genetic resistance (Spadaro and Gullino, 2022). These *formae speciales* can be subdivided into physiological races which are defined by the virulence patterns of susceptible or resistant varieties of the host (Ma et al., 2013). Physiological races can be differentiated from one another by physiological features, such as pathogenicity (Gordon and Martyn, 1997). Molecular tools can be used to identify both pathogenic strains of *Fusarium* spp. and, in some cases, physiological races of the pathogen. Many *formae speciales* have been named based on their host plant, using either species name or genus name (Bahadur, 2021).

Fusarium species cause vascular wilts, rot, canker, leaf spot, and damping-off diseases in a wide range of hosts (Bodah, 2017). Among these, Fusarium wilt is one of the most important disease of vegetables. As previously mentioned, Fusarium wilt is primarily caused by *Fusarium* spp., comprising several important pathogenic species that can infect a variety of hosts, thus causing wilts in crops of economic importance (Gilardi et al., 2017). This fungal pathogen is considered to be among the top ten most important ones in terms of both economic and scientific significance (Yu et al., 2021). Currently, more than 100 *formae speciales* cause disease in a wide range of vegetables (Bahadur, 2021). For example, Fusarium wilt of tomatoes (*F. oxysporum* f. sp. *lycopersici*), Fusarium wilt of lettuce (*F. oxysporum* f. sp. *lactucae*), Fusarium wilt of cultivated and wild rockets (*F. oxysporum* f. sp. *raphani*), Fusarium wilt of spinach (*F. oxysporum* f. sp. *spinaciae*), Fusarium wilt of cabbage (*F. oxysporum* f. sp. *conglutinans*), and Fusarium wilt of cucumber (*F. oxysporum* f. sp. *cucumerinum*) (Gilardi et al., 2007; Ling et al., 2022; Malbrán et al., 2020).

The symptoms of Fusarium wilt vary depending on the infected plant species. However, common symptoms of Fusarium wilt are stunted growth, which is common or sometimes severe, yellowing, and wilting of older leaves. The vascular system turned reddish-brown, which is visible inside the stem as lines, while white, orange, or pink fungal growth appeared outside the stem and eventually decayed

(Subbarao et al., 2017). The severity and extent of damage to the leaves in the field are determined by various factors such as distribution of the inoculum, damage of vascular tissue in the stem, fungal attack duration, and ability of the plant to regrow or retain leaves. The identification of Fusarium wilt at an early stage can be challenging because its early symptoms might be similar to diseases of seedlings, and at later stages may resemble with other diseases (Gordon and Koike, 2015).

Taxonomy of fungal pathogens

Molecular tools (DNA Barcode, MLST and other gene loci)

Characterization of fungal pathogens is challenging due to their high diversity and similar morphological characteristics (Jayawardena et al., 2021). It is more problematic during culturing of fungal isolates. Moreover, morphological approaches, although the cornerstone of fungal diseases, can lead to the unreliable results due to the problems in identification (Hariharan et al., 2021). Because of these issues, molecular-based identification tools are widely used by plant disease diagnosticians and mycologists (Jayawardena et al., 2021). For the fungal molecular identification, an increasingly popular method of using short DNA sequences (500-800 bp), known as DNA barcodes, was recommended (Prakash et al., 2017). Mycologist has adopted internal transcribed spacer (ITS) as primary barcode to identify the fungal pathogens to a reasonable confidence level (Schoch et al., 2012). In general, DNA barcodes must be highly specific for the identification of pathogens. Due to the presence of multiple copies and sequence homology resulting from the parallel evolution of genomic regions make it difficult to construct an accurate phylogenetic tree using a single locus (Raja et al., 2017). In cases where single gene proves insufficient for taxonomic differentiation, multi-locus sequence typing (MLST) has been employed by mycologist for the identification of fungal pathogens and by taxonomists for phylogenetic tree development (Yu et al., 2023).

It helps to recognize species as clades of individuals that are genetically isolated in nature uncovered new cryptic species in many plants pathogenic and toxigenic fungi (Desoubeaux et al., 2023). Additional markers, including the largest and second-largest subunits of RNA polymerase II, translation elongation factor 1-alpha, and beta-tubulin coding genes, are utilized to achieve the resolution needed for species-level classification of fungi and to quantify genetic distances among fungal groups (Raja et al., 2017). However, discriminatory power of a specific MLST scheme is dependent on the choice of the gene loci and accuracy of sequences (Pasic et al., 2020).

Taxonomy of *Alternaria* genus

Description of *Alternaria* spp.

The genus *Alternaria* was first introduced in 1816 by Nees, with *A. tenuis* (type species) from dead plant material (Nees, 1816). The discussion about *Alternaria* taxonomy started from its introduction, and the name of *A. tenuis* (type species of *Alternaria*) was changed to *A. alternata* due to its ambiguous nomenclature (Von Keissler, 1912). The identification *Alternaria* spp. can be difficult due to the huge variation in morphology (spore structure) and their complex nature (Patriarca, 2016).

Taxonomy of *Alternaria* based on Morphological description

Historically, the taxonomy of the *Alternaria* spp. was based on morphological characters, such as mycelial growth of fungi on colony, pattern of sporulation, and characteristics of conidia including size and shape of the conidia, beak size, conidia chain branching patterns and septation (Patriarca, 2016; Simmons, 2007). The taxonomy of the *Alternaria* genus has long been a controversial issue. Simmons (1992) introduced various species groups within *Alternaria* genus based on colony

and conidial morphology to get the unified taxonomic concepts. At the same time, Simmons and Roberts (1993) introduced the concept of species groups and *Alternaria* were divided into six morphological groups. Later, Simmons (2007) revised the *Alternaria* taxonomy and arranged the genus into several groups, which were typified by representative species of *Alternaria* species. Since then, over 1100 species and 275 names have been described based on these morphological characteristics (Simmons, 2007; Somma et al., 2019). Repeated revisions within genus have resulted in the species boundaries being unclear (Puvača et al., 2020). Identification of the *Alternaria* at species level remains challenging due to incorrect taxonomic keys based on morphology (Chethana et al., 2021; Puvača et al., 2020). Moreover, these morphological characteristics are limited in effectiveness because of the overlapping spore characteristics and variability in the size, shape, and septation of conidia between closely related species, making it challenging to establish and define distinct species boundaries (Chethana et al., 2021; Tymon et al., 2016). Therefore, identification based on morphological characters might lead to misidentification. Although the designation of species boundaries within *Alternaria* has not yet been resolved, the concept of morphological groups provides a structure for studying the phylogeny of *Alternaria* at an advanced level (DeMers, 2022).

Taxonomy of *Alternaria* based on Molecular description

Recently, molecular phylogenetic approaches have been widely used, along with morphology, to identify and classify *Alternaria* spp. (Andrew et al., 2009; Peever et al., 2004). Various attempts have been made to resolve the phylogenies of the *Alternaria* section. Molecular phylogenetic studies of *Alternaria* species have shown that they are grouped into numerous distinct phylogenetic lineages, which do not always correspond to clades based on morphological features (Logrieco et al., 2009). Molecular markers such as actin, beta-tubulin (tub2), chitin synthase,

mitochondrial large-subunit (mtLSU), ribosomal DNA, 1,3,8-trihydroxynaphthalene reductase (THNR), and translation elongation factor-1α (*tef-1α*) failed to resolve the species in *Alternaria* section (Peever et al., 2004; Pryor and Gilbertson, 2000). ATPase, Alt-a1, endoPG, SCAR markers, and some anonymous regions were more informative and gave more stable classification with section *Alternaria*, but these loci also failed to discriminate species between groups of small spored *Alternaria* (Ozkilinc et al., 2018; Woudenberg et al., 2013; Zhu and Xiao, 2015). Later, multigene analysis, and whole genome sequencing of nine genes, including Alt-a1, OPA10-2, endoPG, GAPDH gene, ITS, mtLSU, mtSSU, *rpb2*, and *tef-1α* were used and thirty-five morpho-species of *Alternaria* section *Alternaria* were synonymous with *A. alternata* (Woudenberg et al., 2015).

Taxonomy of the *Diaporthe* species based on morpho-molecular description

The genus *Diaporthe* was first recognized as Diaporthaceae by Nitschke (1870). The asexual morph of the genus, *Phomopsis* (Sacc.) Bubák was later introduced in 1905 based on the International Code for Nomenclature of Algae, Fungi and Plant (Index Fungorum, 2023). The Diaporthaceae family was officially established by von Höhnel in 1917 and was subsequently placed within the order Diaporthales, which currently comprises over 50 genera and 32 accepted families (Mena et al., 2023; Wijayawardene et al., 2022). Since elimination of dual nomenclature of fungi, *Diaporthe* and *Phomopsis* are not used to differentiate the different morphs of this genus (Guarnaccia and Crous, 2018). Rossman et al., (2015) recommended that the name of genus *Diaporthe* should be retained on Phomopsis because it was introduced first, represents the most number of species, and therefore has priority.

The taxonomy of *Diaporthe* spp. has been revised in numerous studies (Gomes et al., 2013; Guarnaccia and Crous, 2018; Hilário et al., 2021; Udayanga et al., 2015). To date, over 1200 *Diaporthe* spp. and approximately 1000 names of the genus

Phomopsis have been recorded in the Fungorum (Index Fungorum, 2023). Several *Diaporthe* spp. can be associated with a single host, whereas different host plants are commonly infected by single species of *Diaporthe* (Chaisiri et al., 2021; Gomes et al., 2013). In the past, identification of *Diaporthe* spp. was based on host specificity, cultural features, and morphological characters (shape of ascomata and conidiomata) (Jiang et al., 2021; Santos et al., 2017; Udayanga et al., 2011). However, recent studies have shown that morphological characters are not always trustworthy for the identification of *Diaporthe* spp. at the species level because of association of several *Diaporthe* species with the same host, overlapping morphological traits and their variability in varying environmental conditions (Díaz et al., 2021; Guarnaccia et al., 2018; Norphanphoun et al., 2023). Therefore, molecular-based methods have been used to solve the *Diaporthe* taxonomy (Yang et al., 2018; Guarnaccia et al., 2018). Recently, taxonomy of *Diaporthe* spp. have been resolved using polyphasic protocols including multi-gene phylogenetic and morphological analysis (Bai et al., 2023; Gao et al., 2021; Gomes et al., 2013; Guarnaccia et al., 2018; Yang et al., 2018; Zhu et al., 2023). Multi locus phylogenetic analysis based on sequences of beta-tubulin (TUB), calmodulin (CAL), histone H3 (HIS), nuclear ribosomal internal transcribed spacer (ITS), and translation elongation factor 1-alpha (*tef-1α*) along with morphological characters used to classify the *Diaporthe* spp. (Bai et al., 2023; Dissanayake et al., 2020, Gao et al., 2021; Norphanphoun et al., 2023). Using this polyphasic taxonomic approach, the delimitation of species in the *Diaporthe* genus has significantly improved (Guo et al., 2020). Furthermore, few issues about species boundaries of the *Diaporthe* species complex in were also well resolved, such as the *D. eres* species complex being investigated and identified as a single species by using genealogical concordance methods (Hilário et al., 2021; Norphanphoun et al., 2023).

Taxonomy of the *Fusarium* spp. based on morphology and molecular characteristics

The taxonomic history of the *Fusarium* genus began when it was originally defined as *Fusisporium* based on the type *Fusisporium roseum* by Link in 1809 for fungi with banana- or canoe-shaped conidia (Summerell et al., 2010; Nelson et al., 1981). *Fusarium* spp. are filamentous and naturally ubiquitous species that are primarily found in air and soil. *Fusarium* spp. are the most important fungal species that can directly cause diseases in humans, domesticated animals, and plants (Fourie et al., 2011; Leslie and Summerell, 2006; Logrieco et al., 2003). *F. roseum* was reclassified as *F. graminearum* and *F. sambucinum*, and *F. sambucinum* is now accepted as a type species for *the Fusarium* genus (Wollenweber and Reinking, 1983). The Wollenweber and Reinking classification system remains the foundation for the identification of *Fusarium* spp. (Leslie and Summerell, 2006).

Fusarium spp. are characterized based on the morphological characteristics, such as chlamydospores, presence of microconidia, dimensions and shape of the macroconidia, and pigmentation. *Fusarium* spp. exhibit a high degree of variability in culture (texture and color of colony, cultural aroma), which can be influenced by environmental conditions (Ekwomadu and Mwanza, 2023). Moreover, the classification of *Fusarium* genus continues to undergo major changes due to unclear boundaries based on morphological characteristics. Recently, several phylogenetic studies have shown that species recognition based on morphology often fails to differentiate between many *Fusarium* spp. especially for distinguishing closely related species (Dongzhen et al., 2020; Rana et al., 2022).

Identification and characterization of *Fusarium* isolates based on morphological characteristics alone could be problematic, it is still helpful and commonly used integration with phylogenetic analysis and morphology (Geiser et al., 2004; Hughes et al., 2013; Leslie and Summerell, 2006). Currently, multi-locus phylogenetic analysis is frequently used for the correct identification and distinguish the species

boundaries in *Fusarium* genus (Edwards et al., 2016; Poletto et al., 2020). Lombard et al., (2019) described 15 new cryptic taxa within *Fusarium oxysporum* species complex (FOSC), by using multi-locus phylogenetic and morphology. Further examples of molecular techniques are AFLP, RAPD, *tub2*, *cmda*, IGS, ITS, *tef-1α*, and *rpb2* have been used for diagnosis of fungal strains (Ekwomadu and Mwanza, 2023; Lombard et al., 2015; Rana et al., 2022).

Despite the historical and lack of consensus among *Fusarium* taxonomists, the use of morphological characteristics combined with molecular data improves the accuracy of identification of *Fusarium* isolates. As more research will be conducted it is expected that more accurate taxonomic system likely to be developed (Babadoost, 2018).

References

Abata, L. K., Paz, I. A., Viera, W., & Flores, F. J. (2016). First report of Alternaria rot caused by *Alternaria alternata* on peach in Ecuador. Plant Disease, 100(11), 2323-2323. doi: 10.1094/PDIS-03-16-0318-PDN.

Ahmad, T., Liu, Y., Shujian, H., & Moosa, A. (2020). First record of *Alternaria alternata* causing post-harvest fruit rot of sweet cherry (*Prunus avium*) in China. Plant Disease, 104(7), 2030-2030. doi: 10.1094/PDIS-11-19-2322-PDN.

Al-Rahbi, B. A. A., Al-Sadi, A. M., Al-Mahmooli, I. H., Al-Maawali, S. S., Al-Mahruqi, N. M. T., & Velazhahan, R. (2021). *Meyerozyma guilliermondii* SQUCC-33Y suppresses post-harvest fruit rot of strawberry caused by *Alternaria alternata*. Australasian Plant Pathology, 50, 349-352. doi: 10.1007/s13313-021-00779-z.

Andrew, M., Peever, T. L., & Pryor, B. M. (2009). An expanded multilocus phylogeny does not resolve morphological species within the small-spored *Alternaria* species complex. Mycologia, 101(1), 95-109. doi: 10.3852/08-135.

Ariyawansa, H. A., Tsai, I., Wang, J. Y., Withee, P., Tanjira, M., Lin, S. R., ... & Cheewangkoon, R. (2021). Molecular phylogenetic diversity and biological characterization of *Diaporthe* species associated with leaf spots of *Camellia sinensis* in Taiwan. Plants, 10(7), 1434. doi: 10.3390/plants10071434.

Babadoost, M. (2018). *Fusarium*: Historical and continued importance. *Fusarium*-Plant Diseases, Pathogen Diversity, Genetic Diversity, Resistance and Molecular Markers. doi: 10.5772/intechopen.74147.

Bahadur, A. (2021). Current Status of *Fusarium* and their management strategies. In *Fusarium*-An Overview of the Genus. Intech Open.

Bai, Y., Lin, L., Pan, M., & Fan, X. (2023). Studies of *Diaporthe* (Diaporthaceae, Diaporthales) species associated with plant cankers in Beijing, China, with

three new species described. MycoKeys, 98, 59. doi: 10.3897/mycokeys.98.104156.

Battilani, P., Chiusa, G., Arciuolo, R., Somenzi, M., Fontana, M., Castello, G., & Spigolon, N. (2018). *Diaporthe* as the main cause of hazelnut defects in the Caucasus region. Phytopathologia Mediterranea, 57(2), 320-333. https://doi.org/10.14601/Phytopathol_Mediterr-22872.

Bertetti, D., Guarnaccia, V., Spadaro, D., & Gullino, M. L. (2018). First report of fruit rot in European pear caused by *Diaporthe eres* in Italy. Plant Disease, 102(12), 2651-2651. doi: 10.1094/PDIS-03-18-0499-PDN.

Bodah, E. T. (2017). Root rot diseases in plants: a review of common causal agents and management strategies. Agric. Res. Technol. Open Access J, 5, 555661. doi:10.19080/ARTOAJ.2017.04.555661.

Cancino, S., Lolas, M. A., Galdós, L., Hernández, Y., Ferrada, E., Riveros, P., ... & Díaz, G. A. (2023). Occurrence of *Alternaria alternata* and *A. tenuissima* causing black rot in cherry fruits (*Prunus avium*) in Central Chile. Plant Disease, 107(12), 4032. doi: 10.1094/PDIS-07-23-1435-PDN.

Cara, M., Toska, M., Frasheri, D., Baroncelli, R., & Sanzani, S. M. (2022). *Alternaria* species causing pomegranate and citrus fruit rots in Albania. Journal of Plant Diseases and Protection, 129(5), 1095-1104. doi: 10.1007/s41348-022-00630-7.

Chaisiri, C., Liu, X. Y., Yin, W. X., Luo, C. X., & Lin, Y. (2021). Morphology characterization, molecular phylogeny, and pathogenicity of *Diaporthe passifloricola* on Citrus reticulata cv. Nanfengmiju in Jiangxi Province, China. Plants, 10(2), 218. doi: 10.3390/plants10020218.

Chethana, K. T., Manawasinghe, I. S., Hurdeal, V. G., Bhunjun, C. S., Appadoo, M. A., Gentekaki, E., ... & Hyde, K. D. (2021). What are fungal species and how to delineate them?. Fungal Diversity, 109(1), 1-25. doi: 10.1007/s13225-021-00483-9.

DeMers, M. (2022). *Alternaria alternata* as endophyte and pathogen. Microbiology, 168(3), 001153. doi: 10.1099/mic.0.001153

DeShields, J. B., & Kc, A. N. (2021). Morphological and molecular characterization of *Alternaria* spp. Isolated from European Pears. Plant Disease, 105(9), 2531-2540. doi: 10.1094/PDIS-10-20-2143-RE.

Desoubeaux, G., Debourgogne, A., Wiederhold, N. P., Zaffino, M., Sutton, D., Burns, R. E., ... & Cray, C. (2018). Multi-locus sequence typing provides epidemiological insights for diseased sharks infected with fungi belonging to the *Fusarium solani* species complex. Medical mycology, 56(5), 591-601. doi: 10.1093/mmy/myx089.

Díaz, G. A., Latorre, B. A., Lolas, M., Ferrada, E., Naranjo, P., & Zoffoli, J. P. (2017). Identification and characterization of *Diaporthe ambigua*, *D. australafricana*, *D. novem*, and *D. rudis* causing a post-harvest fruit rot in kiwifruit. Plant Disease, 101(8), 1402-1410. doi: 10.1094/PDIS-10-16-1535-RE.

Díaz, G. A., Zoffoli, J. P., Ferrada, E. E., & Lolas, M. (2021). Identification and pathogenicity of *Diplodia*, *Neofusicoccum*, *Cadophora*, and *Diaporthe* species associated with cordon dieback in kiwifruit cultivar Hayward in Central Chile. Plant Disease, 105(05), 1308-1319. doi: 10.1094/PDIS-05-20-0988-RE.

Dissanayake, A. J., Phillips, A. J. L., Hyde, K. D., Yan, J. Y., & Li, X. H. (2017). The current status of species in *Diaporthe*. Mycosphere 8 (5): 1106–1156. doi: 10.5943/mycosphere/8/5/5.

Dongzhen, F., Xilin, L., Xiaorong, C., Wenwu, Y., Yunlu, H., Yi, C., ... & Chunsheng, G. (2020). *Fusarium* species and *Fusarium oxysporum* species complex genotypes associated with yam wilt in South-Central China. Frontiers in microbiology, 11, 1964. doi: 10.3389/fmicb.2020.01964.

Edel-Hermann, V., & Lecomte, C. (2019). Current status of *Fusarium oxysporum formae speciales* and races. Phytopathology, 109(4), 512-530. doi: 10.1094/PHYTO-08-18-0320-RVW.

Edwards, J., Auer, D., de Alwis, S. K., Summerell, B., Aoki, T., Proctor, R. H., ... & O'Donnell, K. (2016). *Fusarium agapanthi* sp. nov., a novel bikaverin and fusarubin-producing leaf and stem spot pathogen of *Agapanthus praecox* (African lily) from Australia and Italy. Mycologia, 108(5), 981-992. doi: 10.3852/15-333.

Ekwomadu, T. I., & Mwanza, M. (2023). *Fusarium* Fungi Pathogens, Identification, Adverse Effects, Disease Management, and Global Food Security: A Review of the Latest Research. Agriculture, 13(9), 1810. doi. 10.3390/agriculture13091810.

El-Beltagi, H. S., Parmar, A., Chen, T., & El-Mogy, M. M. (2023). Advances in pre- and post-harvest applications to reduce qualitative and quantitative food loss and waste. Frontiers in Plant Science, 14, 1149358. doi: 10.3389/fpls.2023.1149358.

Elfar, K., Bustamante, M. I., Arreguin, M., Nouri, M. T., & Eskalen, A. (2024). Identification and Pathogenicity of Fungal Pathogens Associated with Branch Canker and Shoot Blight on Persimmons (*Diospyros kaki*) in California. Plant Disease, (ja). doi: 10.1094/PDIS-12-23-2629-SC.

Emanuel, I. B., Laird, A. E., & Hand, F. P. (2023). Understanding Environmental and Physiological Factors Affecting the Biology of *Diaporthe ilicicola*, the Fungus Causing Latent Fruit Rot in Winterberry. Plant Disease, 107(10), 2986-2996. doi: 10.1094/PDIS-11-22-2759-RE.

FAO. (January 25, 2023). Leading producers of fresh vegetables worldwide in 2021 (in million metric tons) [Graph]. In Statista. Retrieved February 05, 2024, from https://www.statista.com/statistics/264662/top-producers-of-fresh-vegetables-worldwide/.

FAO. (January 6, 2023). Leading producers of fresh fruit worldwide in 2021 (in million metric tons)* [Graph]. In Statista. Retrieved February 05, 2024, from https://www.statista.com/statistics/279164/global-top-producers-of-selected-fresh-fruit-worldwide/.

FAO. (2021). Leading producers of fresh fruit worldwide in 2021 (in million metric tons).

Fourie, G., Steenkamp, E. T., Ploetz, R. C., Gordon, T. R., & Viljoen, A. (2011). Current status of the taxonomic position of *Fusarium oxysporum formae specialis cubense* within the *Fusarium oxysporum* complex. Infection, Genetics and Evolution, 11(3), 533-542. doi: 10.1016/j.meegid.2011.01.012.

Gao, H., Pan, M., Tian, C., & Fan, X. (2021). *Cytospora* and *Diaporthe* species associated with hazelnut canker and dieback in Beijing, China. Frontiers in Cellular and Infection Microbiology, 11. doi: 10.3389/fcimb.2021.664366.

Geiser, D. M., del Mar Jiménez-Gasco, M., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T. J., ... & O'donnell, K. (2004). FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. European journal of plant pathology, 110, 473-479. doi: 10.1023/B:EJPP.0000032386.75915.a0.

Geng, M., & Wang, J. (2023). First Report of Black Rot on Persimmon Fruits Caused by *Diaporthe eres* in China. Plant Disease, 107(10), 3292. doi: 10.1094/PDIS-03-23-0601-PDN.

Gilardi, G., Chen, G., Garibaldi, A., Zhiping, C., & Gullino, M. L. (2007). Resistance of different rocket cultivars to wilt caused by strains of *Fusarium oxysporum* under artificial inoculation conditions. Journal of Plant Pathology, 113-117. https://www.jstor.org/stable/41998364.

Gilardi, G., Franco Ortega, S., Van Rijswick, P. C. J., Ortu, G., Gullino, M. L., & Garibaldi, A. (2017). A new race of *Fusarium oxysporum* f. sp. lactucae of lettuce. Plant Pathology, 66(4), 677-688. doi: 10.1111/ppa.12616.

Gomes, R. R., Glienke, C., Videira, S. I. R., Lombard, L., Groenewald, J. Z., & Crous, P. W. (2013). *Diaporthe*: a genus of endophytic, saprobic and plant pathogenic fungi. Persoonia-Molecular Phylogeny and Evolution of Fungi, 31(1), 1-41. doi: 10.3767/003158513X666844.

Gordon, T. R., 2017. F. oxysporum and the Fusarium wilt syndrome. Annual review of phytopathology, 55, 23–39. doi: 10.1146/annurev-phyto-080615-095919.

Gordon, T. R., & Koike, S. T. (2015). Management of Fusarium wilt of lettuce. Crop Protection, 73, 45-49. doi: 10.1016/j.cropro.2015.01.011.

Gordon, T. R., & Martyn, R. D. (1997). The evolutionary biology of *Fusarium oxysporum*. Annual review of phytopathology, 35(1), 111-128. doi: 10.1146/annurev.phyto.35.1.111.

Guarnaccia, V., & Crous, P. W. (2018). Species of *Diaporthe* on camellia and citrus in the Azores Islands. Phytopathologia Mediterranea, 57(2), 307-319. doi: 10.14601/Phytopathol_Mediterr-23254.

Guarnaccia, V., Groenewald, J. Z., Woodhall, J., Armengol, J., Cinelli, T., Eichmeier, A., ... & Crous, P. W. (2018). *Diaporthe* diversity and pathogenicity revealed from a broad survey of grapevine diseases in Europe. Persoonia-Molecular Phylogeny and Evolution of Fungi, 40(1), 135-153. doi: 10.3767/persoonia.2018.40.06.

Guo, L. W., He, S. H., Gao, Z. R., Wu, Z. T., Duan, R. Q., Yang, K. Z., ... & He, X. H. (2020). First Report of Fruit Rot in Opium Poppy (*Papaver somniferum*) Caused by *Alternaria alternata* in China. Plant Disease, 104(12), 3264. doi: 10.1094/PDIS-01-20-0211-PDN.

Hariharan, G., & Prasannath, K. (2021). Recent advances in molecular diagnostics of fungal plant pathogens: A mini review. Frontiers in Cellular and Infection Microbiology, 10, 600234. doi: 10.3389/fcimb.2020.600234.

Hasan, N. A., & Zanuddin, N. A. M. (2018). Molecular identification of isolated fungi from banana, mango and pineapple spoiled fruits. In AIP Conference Proceedings (Vol. 2020, No. 1). AIP Publishing. doi: 10.1063/1.5062700.

He, J., Li, D. W., Cui, W. L., & Huang, L. (2024). Seven new species of *Alternaria* (Pleosporales, Pleosporaceae) associated with Chinese fir, based on morphological and molecular evidence. MycoKeys, 101, 1. doi: 10.3897/mycokeys.101.115370.

Hilário, S., Gonçalves, M. F., & Alves, A. (2021). Using genealogical concordance and coalescent-based species delimitation to assess species boundaries in the *Diaporthe eres* complex. Journal of Fungi, 7(7), 507. doi: 10.3390/jof7070507.

Huang, F., Hou, X., Dewdney, M. M., Fu, Y., Chen, G., Hyde, K. D., & Li, H. (2013). *Diaporthe* species occurring on citrus in China. Fungal Diversity, 61, 237-250. doi: 10.1007/s13225-013-0245-6.

Hughes, K. W., Petersen, R. H., Lodge, D. J., Bergemann, S. E., Baumgartner, K., Tulloss, R. E., ... & Cifuentes, J. (2013). Evolutionary consequences of putative intra-and interspecific hybridization in agaric fungi. Mycologia, 105(6), 1577-1594. doi: 10.3852/13-041.

Index Fungorum, (2023). Index fungorum. Index fungorum. Available online at: http://www.indexfungorum.org/names/Names.asp (accessed October, 2023)

Jayawardena, R. S., Hyde, K. D., de Farias, A. R. G., Bhunjun, C. S., Ferdinandez, H. S., Manamgoda, D. S., ... & Thines, M. (2021). What is a species in fungal plant pathogens?. Fungal Diversity, 109(1), 239-266. doi: 10.1007/s13225-021-00484-8.

Jiang, N., Voglmayr, H., Piao, C. G., & Li, Y. (2021). Two new species of *Diaporthe* (Diaporthaceae, Diaporthales) associated with tree cankers in the Netherlands. MycoKeys, 85, 31. doi: 10.3897/mycokeys.85.73107.

Jianying, F., Bianyu, Y., Xin, L., Dong, T., & Weisong, M. (2021). Evaluation on risks of sustainable supply chain based on optimized BP neural networks in fresh grape industry. Computers and Electronics in Agriculture, 183, 105988. doi: 10.1016/j.compag.2021.105988.

Kc, A. N., & Rasmussen, A. L. (2019). First report of *Diaporthe rudis* causing fruit rot of European pears in the United States. Plant Disease, 103(8), 2132-2132. doi: 10.1094/PDIS-12-18-2184-PDN.

Keissler, K. Von. (1912). Zur kenntnis der pilzflora krains. Beihefte zum Botanischen Zentralblatt. 29, 395-440.

Khuna, S., Kumla, J., Thitla, T., Nuangmek, W., Lumyong, S., & Suwannarach, N. (2022). Morphology, molecular identification, and pathogenicity of two novel *Fusarium* species associated with post-harvest fruit rot of cucurbits in Northern Thailand. Journal of Fungi, 8(11), 1135. doi: 10.3390/jof8111135.

Latinović, N., Radišek, S., & Latinović, J. (2014). First report of *Alternaria alternata* causing fruit rot on fig (*Ficus carica*) in Montenegro. Plant disease, 98(3), 424-424. doi: 10.1094/PDIS-07-13-0770-PDN.

Lawrence, D. P., Rotondo, F., & Gannibal, P. B. (2016). Biodiversity and taxonomy of the pleomorphic genus *Alternaria*. Mycological progress, 15, 1-22. doi: 10.1007/s11557-015-1144-x.

Leslie, J. F. (2012). Genetics and *Fusarium oxysporum*. In: Gullino, M. L., Katan, J., Garibaldi, A. (eds) Fusarium wilts of greenhouse vegetable and ornamental crops. The American Phytopathological Society, St. Paul, pp 39-46. doi: 10.1094/9780890544822.

Leslie, J. F. and Summerell, B.A. (2006). The *Fusarium* Laboratory Manual. Oxford: Blackwell Publishing.

Li, J. F., Jiang, H. B., Jeewon, R., Hongsanan, S., Bhat, D. J., Tang, S. M., ... & Phookamsak, R. (2023). *Alternaria*: Update on species limits, evolution, multi-locus phylogeny, and classification. Studies in Fungi, 8(1), 1-61. doi: 10.48130/SIF-2023-0001.

Li, D., Zhang, X., Gu, X., Zhang, Q., Zhao, L., Zheng, X., & Zhang, H. (2019). The infection of grapes by *Talaromyces rugulosus* O1 and the role of cell wall-degrading enzymes and ochratoxin A in the infection. Physiological and Molecular Plant Pathology, 106, 263-269. doi: 10.1016/j.pmpp.2019.03.006.

Li, L., Pan, H., Liu, W., Chen, M. Y., & Zhong, C. H. (2017). First report of *Alternaria alternata* causing post-harvest rot of kiwifruit in China. Plant Disease, 101(6), 1046. doi: 10.1094/PDIS-11-16-1611-PDN.

Ling, J., Dong, X., Ping, X., Li, Y., Yang, Y., Zhao, J., ... & Mao, Z. (2022). Genetic Diversity and Population Structure of *Fusarium oxysporum* f. sp. *conglutinans* Race 1 in Northern China Samples. Journal of Fungi, 8(10), 1089. doi: 10.3390/jof8101089.

Logrieco, A., Moretti, A., & Solfrizzo, M. (2009). *Alternaria* toxins and plant diseases: an overview of origin, occurrence and risks. World Mycotoxin Journal, 2(2), 129-140. doi: 10.3920/WMJ2009.1145. doi: 10.1094/PDIS-10-18-1757-PDN.

Logrieco, A., Bottalico, A., Mulé, G., Moretti, A., & Perrone, G. (2003). Epidemiology of toxigenic fungi and their associated mycotoxins for some Mediterranean crops. Epidemiology of Mycotoxin Producing Fungi: Under the aegis of COST Action 835 'Agriculturally Important Toxigenic Fungi 1998–2003', EU project (QLK 1-CT-1998–01380), 645-667. doi: 10.1023/A:1026033021542.

Lombard, L., Sandoval-Denis, M., Lamprecht, S. C., & Crous, P. W. (2019). Epitypification of *Fusarium oxysporum*–clearing the taxonomic chaos.

Persoonia-Molecular Phylogeny and Evolution of Fungi, 43(1), 1-47. doi: 10.3767/persoonia.2019.43.01.

Lombard L, Van der Merwe NA, Groenewald JZ, et al. 2015. Generic concept of Nectriaceae. Studies in Mycology 80: 189–245. doi: 10.1016/j.simyco.2014.12.002.

Lorenzini, M., & Zapparoli, G. (2019). *Diaporthe rudis* associated with berry rot of post-harvest grapes in Italy. Plant Disease, 103(5), 1030-1030. doi: 10.1094/PDIS-10-18-1757-PDN.

Malbrán, I., Mourelos, C. A., & Lori, G. A. (2020). First report of *Fusarium oxysporum* f. sp. *lycopersici* race 3 causing Fusarium wilt of tomato in Argentina. Plant Disease, 104(3), 978-978. doi: 10.1094/PDIS-08-19-1777-PDN.

Manjunatha, N., Sharma, J., Pokhare, S. S., Agarrwal, R., Patil, P. G., Sirsat, J. D., ... & Marathe, R. A. (2022). Characterization of *Alternaria* and *Colletotrichum* species associated with pomegranate (*Punica granatum* L.) in Maharashtra state of India. Journal of Fungi, 8(10), 1040. doi: 10.3390/jof8101040.

Miranda-Apodaca, J., Artetxe, U., Aguado, I., Martin-Souto, L., Ramirez-Garcia, A., Lacuesta, M., ... & Pérez-López, U. (2023). Stress Response to Climate Change and Post-harvest Handling in Two Differently Pigmented Lettuce Genotypes: Impact on *Alternaria alternata* Invasion and Mycotoxin Production. Plants, 12(6), 1304. doi: 10.3390/plants12061304.

Mostafa, A. M., Kumar, S. A., Meraj, T., Rauf, H. T., Alnuaim, A. A., & Alkhayyal, M. A. (2021). Guava disease detection using deep convolutional neural networks: A case study of guava plants. Applied Sciences, 12(1), 239. doi: 10.3390/app12010239.

Moura, S. C. S. R. D., & Vialta, A. (2022). Use of fruits and vegetables in processed foods: consumption trends and technological impacts. Food Science and Technology, 42, e66421. doi:10.1590/fst.66421.

Nees von Esenbeck, C. G. (n.d.) 1816 ("1817" in some copies). Das System der Pilze und Schwamme:72.

Nelson, P. E., Toussoun, T. A., Cook, R. J. (1981). *Fusarium*: Diseases, Biology, and Taxonomy; The Pennsylvania State University: State College, PA, USA, 257 pp.

Nitschke, T. (1870). Pyrenomycetes Germanici 2: 245 Breslau. Eduard Trewendt, Germany.

Norphanphoun, C., Gentekaki, E., Hongsanan, S., Jayawardena, R., Senanayake, I. C., Manawasinghe, I. S., ... & Hyde, K. D. (2023). *Diaporthe*: Formalizing the species-group concept. doi: 10.5943/mycosphere/13/1/9.

Ntasiou, P., Myresiotis, C., Konstantinou, S., Papadopoulou-Mourkidou, E., & Karaoglanidis, G. S. (2015). Identification, characterization and mycotoxigenic ability of *Alternaria* spp. causing core rot of apple fruit in Greece. International journal of food microbiology, 197, 22-29. doi: 10.1016/j.ijfoodmicro.2014.12.008.

Olaya, B., Essau, C. A., Moneta, M. V., Lara, E., Miret, M., Martín-María, N., ... & Haro, J. M. (2019). Fruit and vegetable consumption and potential moderators associated with all-cause mortality in a representative sample of Spanish older adults. Nutrients, 11(8), 1794. doi: 10.3390/nu11081794.

Ozkilinc, H., Rotondo, F., Pryor, B. M., & Peever, T. L. (2018). Contrasting species boundaries between sections *Alternaria* and *Porri* of the genus *Alternaria*. Plant Pathology, 67(2), 303-314. doi: 10.1111/ppa.12749.

Pasic, L., Goterris, L., Guerrero-Murillo, M., Irinyi, L., Kan, A., Ponce, C. A., ... & Meyer, W. (2020). Consensus multilocus sequence typing scheme for

Pneumocystis jirovecii. Journal of Fungi, 6(4), 259. doi: 10.3390/jof6040259.

Patriarca, A. (2016). Alternaria in food products. Current Opinion in Food Science, 11, 1-9. doi: 10.1016/j.cofs.2016.08.007.

Peever, T. L., Su, G., Carpenter-Boggs, L., & Timmer, L. W. (2004). Molecular systematics of citrus-associated *Alternaria* species. Mycologia, 96(1), 119-134. doi: 10.1080/15572536.2005.11833002.

Pirozzi, A., Ferrari, G., & Donsì, F. (2021). The use of nanocellulose in edible coatings for the preservation of perishable fruits and vegetables. Coatings, 11(8), 990. doi: 10.3390/coatings11080990.

Porat, R., Lichter, A., Terry, L. A., Harker, R., & Buzby, J. (2018). Post-harvest losses of fruit and vegetables during retail and in consumers' homes: Quantifications, causes, and means of prevention. Post-harvest biology and technology, 139, 135-149. doi: 10.1016/j.postharvbio.2017.11.019.

Prakash, P. Y., Irinyi, L., Halliday, C., Chen, S., Robert, V., & Meyer, W. (2017). Online databases for taxonomy and identification of pathogenic fungi and proposal for a cloud-based dynamic data network platform. Journal of Clinical Microbiology, 55(4), 1011-1024. doi: 10.1128/jcm.02084-16.

Pryor, B. M., & Gilbertson, R. L. (2000). Molecular phylogenetic relationships amongst Alternaria species and related fungi based upon analysis of nuclear ITS and mt SSU rDNA sequences. Mycological Research, 104(11), 1312-1321. doi: 10.1017/S0953756200003002.

Puvača, N., Bursić, V., Vuković, G., Budakov, D., Petrović, A., Merkuri, J., ... & Cara, M. (2020). Ascomycete fungi (*Alternaria* spp.) characterization as major feed grains pathogens. Journal of Agronomy, Technology and Engineering Management, 3(6), 499-505.

Raja, H. A., Miller, A. N., Pearce, C. J., & Oberlies, N. H. (2017). Fungal identification using molecular tools: a primer for the natural products

research community. Journal of natural products, 80(3), 756-770. doi: 10.1021/acs.jnatprod.6b01085.

Ramezani, Y., Taheri, P., & Mamarabadi, M. (2019). Identification of *Alternaria* spp. associated with tomato early blight in Iran and investigating some of their virulence factors. Journal of Plant Pathology, 101, 647-659. doi: 10.1007/s42161-019-00259-w.

Rana, A., Sahgal, M., Johri, B.N. (2017). *Fusarium oxysporum*: Genomics, Diversity and Plant–Host Interaction. In: Satyanarayana, T., Deshmukh, S., Johri, B. (eds) Developments in Fungal Biology and Applied Mycology. Springer, Singapore. doi: 10.1007/978-981-10-4768-8_10. doi: 10.3390/jof8070662.

Rana, S., Singh, S. K., & Dufossé, L. (2022). Multigene Phylogeny, Beauvericin Production and Bioactive Potential of *Fusarium* Strains Isolated in India. Journal of Fungi, 8(7), 662. doi: 10.3390/jof8070662.

Riquelme, D., Zúñiga, C., & Tapia, E. (2021). First Report of Fruit Rot of Sweet Cultivars of Japanese Plum Caused by *Alternaria alternata*, *A. arborescens*, and *A. tenuissima* in Chile. Plant Disease, 105(12), 4167. doi: 10.1094/PDIS-03-21-0465-PDN.

Rossman, A. Y., Crous, P. W., Hyde, K. D., Hawksworth, D. L., Aptroot, A., Bezerra, J. L., ... & Zhang, Y. (2015). Recommended names for pleomorphic genera in Dothideomycetes. IMA fungus, 6, 507-523. doi: 10.5598/imafungus.2015.06.02.14.

Saharan, G. S., Mehta, N., Meena, P. D., & Dayal, P. (2016). *Alternaria* diseases of crucifers: biology, ecology and disease management (pp. 17-51). Singapore: Springer. Doi: 10.1007/978-981-10-0021-8.

Santos, L., Phillips, A. J. L., Crous, P. W., & Alves, A. (2017). *Diaporthe* species on Rosaceae with descriptions of *D. pyracanthae* sp. nov. and *D. malorum* sp. nov. Mycosphere, 8(5), 485-511. doi: 10.5943/mycosphere/8/5/1.

Shipman, E. N., Yu, J., Zhou, J., Albornoz, K., & Beckles, D. M. (2021). Can gene editing reduce post-harvest waste and loss of fruit, vegetables, and ornamentals?. Horticulture research, 8. doi: 10.1038/s41438-020-00428-4.

Singh, D., & Sharma, R. R. (2018). Post-harvest diseases of fruits and vegetables and their management. In Post-harvest disinfection of fruits and vegetables (pp. 1-52). Academic Press. doi: 10.1016/B978-0-12-812698-1.00001-7.

Sivakumar, D., & Bautista-Baños, S. (2014). A review on the use of essential oils for post-harvest decay control and maintenance of fruit quality during storage. Crop protection, 64, 27-37. doi: 10.1016/j.cropro.2014.05.012.

Slathia, S., Sharma, Y. P., Hakla, H. R., Urfan, M., Yadav, N. S., & Pal, S. (2021). Post-harvest Management of *Alternaria* Induced Rot in Tomato Fruits With Essential Oil of *Zanthoxylum armatum* DC. Frontiers in Sustainable Food Systems, 5, 679830. doi: 10.3389/fsufs.2021.679830.

Schoch C., L, Seifert, K. A., Eckert, S. E., Robert, V., Spouge, J. L., Levesque, C., André, et al. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences.109(16):6241–6246. doi: 10.1073/pnas.111701810.

Simmons EG. (2007). *Alternaria*: An identification manual, CBS Biodiversity Series 6. Centraalbureau voor Schimmelcultures, Utrecht.

Simmons, E. G., & Roberts, R. G. (1993). *Alternaria* themes and variations (73). Mycotaxon, 48, 109-140.

Simmons, E. G. (1992). *Alternaria* taxonomy: current status, viewpoint, challenge. *Alternaria* biology, plant diseases and metabolites, 1-35.

Somma, S., Amatulli, M. T., Masiello, M., Moretti, A., & Logrieco, A. F. (2019). *Alternaria* species associated to wheat black point identified through a multilocus sequence approach. International Journal of Food Microbiology, 293, 34-43. doi: 10.1016/j.ijfoodmicro.2019.01.001

Spadaro, D., & Gullino, M. L. (2022). Crosstalk Between Wilt-Causing Fungi, Plants and Their Microbiome. In Microbial Cross-talk in the Rhizosphere (pp. 155-178). Singapore: Springer Nature Singapore. doi: 10.1007/978-981-16-9507-0_7.

Subbarao, K. V., Davis, R. M., Gilbertson, R. L., & Raid, R. N. (2017). Compendium of lettuce diseases and pests (No. Ed. 2). American Phytopathological Society (APS Press). doi: 10.1094/9780890545782.

Summerell, B. A., Laurence, M. H., Liew, E. C., & Leslie, J. F. (2010). Biogeography and phylogeography of *Fusarium*: a review. Fungal Diversity, 44(1), 3-13. doi: 10.1007/s13225-010-0060-2.

Szabó, M., Csikász-Krizsics, A., Dula, T., Farkas, E., Roznik, D., Kozma, P., & Deák, T. (2023). Black Rot of Grapes (*Guignardia bidwellii*)—A Comprehensive Overview. Horticulturae, 9(2), 130. doi: 10.3390/horticulturae9020130.

Tekiner, N., Tozlu, E., & Guarnaccia, V. (2020). First report of *Diaporthe foeniculina* causing fruit rot of lemon in Turkey. Journal of Plant Pathology, 102(1), 277-277. doi: 10.1007/s42161-019-00413-4.

Timmer, L. W., Akimitsu, K., Solel, Z. V. I., & Peever, T. L. (2003). *Alternaria* diseases of citrus-novel pathosystems, Phytopathologia Mediterranean, 42, 99–112. doi: 10.14601/Phytopathol_Mediterr-1710.

Thomidis, T., Prodromou, I., & Zambounis, A. (2019). Occurrence of *Diaporthe ambigua* Nitschke causing post-harvest fruit rot on kiwifruit in Chrysoupoli Kavala, Greece. Journal of Plant Pathology, 101(4), 1295-1296. doi: 10.1007/s42161-019-00356-w.

Tymon, L. S., Peever, T. L., & Johnson, D. A. (2016). Identification and enumeration of small-spored *Alternaria* species associated with potato in the US Northwest. Plant disease, 100(2), 465-472. doi: 10.1094/PDIS-03-15-0263-RE.

Udayanga, D., Castlebury, L. A., Rossman, A. Y., Chukeatirote, E., & Hyde, K. D. (2015). The *Diaporthe sojae* species complex: Phylogenetic re-assessment of pathogens associated with soybean, cucurbits and other field crops. Fungal Biology, 119(5), 383-407. doi: 10.1016/j.funbio.2014.10.009.

Udayanga, D., Liu, X., McKenzie, E. H., Chukeatirote, E., Bahkali, A. H., & Hyde, K. D. (2011). The genus *Phomopsis*: biology, applications, species concepts and names of common phytopathogens. Fungal diversity, 50, 189-225. doi: 10.1007/s13225-011-0126-9.

Vitale, A., Aiello, D., Azzaro, A., Guarnaccia, V., & Polizzi, G. (2021). An eleven-year survey on field disease susceptibility of citrus accessions to *Colletotrichum* and *Alternaria* species. Agriculture, 11(6), 536. doi: 10.3390/agriculture11060536.

Wallace, T. C., Bailey, R. L., Blumberg, J. B., Burton-Freeman, B., Chen, C. O., Crowe-White, K. M., ... & Wang, D. D. (2020). Fruits, vegetables, and health: A comprehensive narrative, umbrella review of the science and recommendations for enhanced public policy to improve intake. Critical reviews in food science and nutrition, 60(13), 2174-2211. doi: 10.1080/10408398.2019.1632258.

Wang, F., Saito, S., Michailides, T. J., & Xiao, C. L. (2021). Phylogenetic, morphological, and pathogenic characterization of *Alternaria* species associated with fruit rot of mandarin in California. Plant Disease, 105(9), 2606-2617. doi: 10.1094/PDIS-10-20-2145-RE.

Wijayawardene, N. N., Hyde, K. D., Dai, D. Q., Sánchez-García, M., Goto, B. T., & Magurno, F. (2022). Outline of Fungi and fungus-like taxa–2021. doi: 10.5943/mycosphere/13/1/2.

Wollenweber, H.W., Reinking, O. A. (1983). Die Usarien, HhreBeschreibung, Schadwirkung, und Bekämpfung; Verlag Paul Parey: Berlin, Germany.

Woudenberg, J. H. C., Seidl, M. F., Groenewald, J. Z., De Vries, M., Stielow, J. B., Thomma, B. P. H. J., & Crous, P. W. (2015). *Alternaria* section *Alternaria*: Species, *formae speciales* or pathotypes?. Studies in mycology, 82(1), 1-21. doi: 10.1016/j.simyco.2015.07.001.

Woudenberg, J. H. C., Groenewald, J. Z., Binder, M., & Crous, P. W. (2013). *Alternaria* redefined. Studies in mycology, 75(1), 171-212. doi: 10.3114/sim0015.

Xiang, M. L., Li, S. C., Wu, F., Zhao, X. Y., Wang, Y. B., An, X. X., ... & Chen, M. (2021). First report of *Alternaria alternata* causing fruit rot on *Tetradium ruticarpum* in China. Plant Disease, 105(4), 1194. doi: 10.1094/PDIS-08-20-1853-PDN.

Xiao, Y., Huo, G., Liu, L., Yang, C., & Cui, C. (2022). First report of post-harvest fruit rot disease of yellow peach caused by *Diaporthe eres* in China. Plant Disease, 106(7), 1983. doi: 10.1094/PDIS-11-21-2484-PDN.

Xiao, Y., Zou, M., Zhou, X., Yang, C., & Cui, C. (2023). First Report of Post-harvest Fruit Brown Rot Disease on Navel Orange Caused by *Diaporthe sojae* in China. Plant Disease, 107(3), 948. doi: 10.1094/PDIS-04-22-0922-PDN.

Yang, Q., Fan, X. L., Guarnaccia, V., & Tian, C. M. (2018). High diversity of *Diaporthe* species associated with dieback diseases in China, with twelve new species described. MycoKeys, (39), 97. doi: 10.3897/mycokeys.39.26914.

Yu, P. L., Fulton, J. C., Hudson, O. H. et al. (2023). Next-generation fungal identification using target enrichment and Nanopore sequencing. BMC Genomics 24: 581. doi: 10.1186/s12864-023-09691-w.

Yu, D. S., Outram, M. A., Smith, A., McCombe, C. L., Khambalkar, P. B., Rima, S. A., ... & Williams, S. J. (2021). The structural repertoire of *Fusarium oxysporum* f. sp. *lycopersici* effectors revealed by experimental and computational studies. BioRxiv, 2021-12. doi: 10.1101/2021.12.14.472499.

Zabiák, A., Kovács, C., Takács, F., Pál, K., Peles, F., Fekete, E., ... & Sándor, E. (2023). *Diaporthe* and *Diplodia* Species Associated with Walnut (*Juglans regia* L.) in Hungarian Orchards. Horticulturae, 9(2), 205. doi: 10.3390/horticulturae9020205.

Zoffoli, J. P., Gil, G. F., & Crisosto, C. G. (1998). Evaluación crítica del manejo de frutos de kiwi desde la cosecha. Aconex, 58, 18-24.

Zhang, Z., Zhang, Z. B., Huang, Y. T., Wang, F. X., Hu, W. H., Dai, L. Y., ... & Zhu, J. Z. (2021). First report of *Diaporthe hongkongensis* causing fruit rot on peach (*Prunus persica*) in China. Plant Disease, 105(7), 2017. doi: 10.1094/PDIS-07-20-1505-PDN.

Zhu, Y. Q., Ma, C. Y., Xue, H., Piao, C. G., Li, Y., & Jiang, N. (2023). Two new species of *Diaporthe* (Diaporthaceae, Diaporthales) in China. MycoKeys, 95, 209. doi: 10.3897/mycokeys.95.98969.

Zhu, X. Q., & Xiao, C. L. (2015). Phylogenetic, morphological, and pathogenic characterization of *Alternaria* species associated with fruit rot of blueberry in California. Phytopathology, 105(12), 1555-1567. doi: 10.1094/PHYTO-05-15-0122-R.

Chapter 2

Journal of *Fungi*

Molecular Characterization and Pathogenicity of *Alternaria* spp. Associated with Black Rot of Sweet Cherries in Italy

Muhammad Waqas [1], Simona Prencipe [1], Vladimiro Guarnaccia [1,2] and Davide Spadaro[1,2*]

[1] Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy; muhammad.waqas@unito.it (M.W.); simona.prencipe@unito.it (S.P.); vladimiro.guarnaccia@unito.it (V.G.)

[2] AGROINNOVA—Interdepartmental Centre for Innovation in the Agro-environmental Sector, University of Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy

[*] Correspondence: davide.spadaro@unito.it; Tel.: +39-011-6708942

Published in Journal of Fungi (2023), https://doi.org/10.3390/jof9100992

Abstract

Black rot is limiting the production of sweet cherries in Italy. Dark brown to black patches and sunken lesions on fruits are the most common symptoms of *Alternaria* black rot on sweet cherry fruits. We isolated 180 *Alternaria* spp. from symptomatic cherry fruits 'Kordia', 'Ferrovia', and 'Regina' harvested in Northern Italy, over three years, from 2020 to 2022. The aim was to identify and characterize a selection of forty isolates of *Alternaria* spp. based on morphology, pathogenicity, and combined analysis of *rpb2*, Alt-a1, endoPG and OPA10-2. The colonies were dark greyish in the center with white margins. Ellipsoidal or ovoid shaped conidia ranging from 19.8 to 21.7 µm in length were observed under a microscope. Based on the concatenated session of four gene regions, thirty-three out of forty isolates were identified as *A. arborescens* species complex (AASC), and seven as *A. alternata*. Pathogenicity was evaluated on healthy 'Regina' sweet cherry fruits. All the tested strains were pathogenic on their host. This study represents the first characterization of *Alternaria* spp. associated with black rot of cherries in Italy and, to the best of our knowledge, it is also the first report of AASC as an agent of black rot of sweet cherries in Italy.

Keywords

Alternaria alternata; *Alternaria arborescens* species complex; *Prunus avium*; phylogeny

1. Introduction

Sweet cherry (*Prunus avium* L.) is an economically important stone fruit which belongs to the genus *Prunus* within the Rosaceae family. Italy is the seventh sweet cherry producer in the world after Turkey, United States of America, Chile, Iran, Uzbekistan, and Spain and the second in Europe [1]. In Italy, sweet cherry is

cultivated on an area of 28,609 ha, with an annual production of approximately 107,905 t [2].

Sweet cherries are highly perishable fruit, with a shelf life of 7–14 days [3] and they are susceptible, during cold storage, to decay caused by postharvest pathogens, including *Monilinia* spp., agent of brown rot; *Botrytis cinerea,* agent of gray mold; *Penicillium expansum*, agent of blue mold; and *Rhizopus* spp., agent of soft rot [4–7]. Cherry production could be threatened also by black rot caused by *Alternaria* species, which is emerging as a major fungal disease [8–10]. Symptoms of black rot of *Alternaria* appear as dark brown to black patches on the outer surface of fruits. These patches gradually increase in size and surface and, under high-humidity conditions, may develop white to light brown, fluffy and moldy growth, and ultimately can cause complete fruit rot [8,11,12].

The genus *Alternaria* was originally described by Nees von Esenbeck in 1816; it is part of the *Pleosporaceae* family [13] and includes endophytic, pathogenic, and saprobic species [14–17]. *Alternaria* spp. are distributed all over the world and may infect over 4000 plant species [13,15,17], such as vegetables, cereals and fruit trees in field and during storage [18–23]. Black rot caused by *A. alternata* (Fr.) Keissl. was recently reported on cherries in Chile [9] and in China [8,10]. *A. alternata* is also associated with brown to black spots on cherry leaves [24,25]. Alternaria rots and spots are becoming more and more frequent on cherry and on other fruit crops due to climate change, characterized by the increase of average temperature, high-humidity conditions and stressed plants, weakened by abiotic stresses. Biological and environmental factors are causing a shift in the fruit microbiological ecology, which is influencing the pre- and postharvest development of *Alternaria* spp. [26].

In the past, *Alternaria* spp. were identified based on macro- and micro-morphological characteristics [16,27]. Nowadays, DNA-based molecular techniques are used to identify *Alternaria* at species level to avoid morphological variations depending on the environmental conditions [13,15,28–32]. Different

molecular approaches have been used for the identification of *Alternaria* section, resulting in several taxonomic revisions [13,16,28,33–37]. Most small-spored *Alternaria* species with concatenated conidia belong to the section Alternaria [16], and *A. alternata* (Fr.) Keissl. and *A. arborescens* Simmons are important representative plant pathogens of this section [16]. To the best of our knowledge, *Alternaria* spp. were not previously reported and characterized as agents of black rot of sweet cherries in Italy.

The aims of this study are to isolate and identify the *Alternaria* species associated with black rot of sweet cherries, based on morphological and phylogenetic analysis, and to confirm their pathogenicity and virulence on sweet cherries.

2. Materials and Methods

2.1. Sampling and Isolation

A monitoring of postharvest diseases was conducted in sweet cherry harvested from orchards located in Piedmont, Northern Italy, from 2020 to 2022. A total of 180 isolates were isolated from symptomatic cherry fruits belonging to the varieties 'Regina', 'Kordia', and 'Ferrovia' in the packinghouses of Piedmont (Figure 1A, B). Sweet cherry fruits were surface disinfected with 1% sodium hypochlorite for 1 min, rinsed in sterile water for 1 min, and dried on sterile filter paper. Then five pieces of black rotten fruits were cut at the margin between healthy and infected tissues and plated on potato dextrose agar (PDA, VWR international, Leuven, Belgium) [29] containing streptomycin (0.025 g/L). The PDA plates were incubated at 22 ± 1 °C for 3 days. Pure cultures were obtained by transferring the mycelium plug from the edge of the colonies and placed in fresh PDA plates. Isolates used in this study were maintained and kept at −80 °C in the culture collection of the University of Turin, Torino, Italy (Table 1).

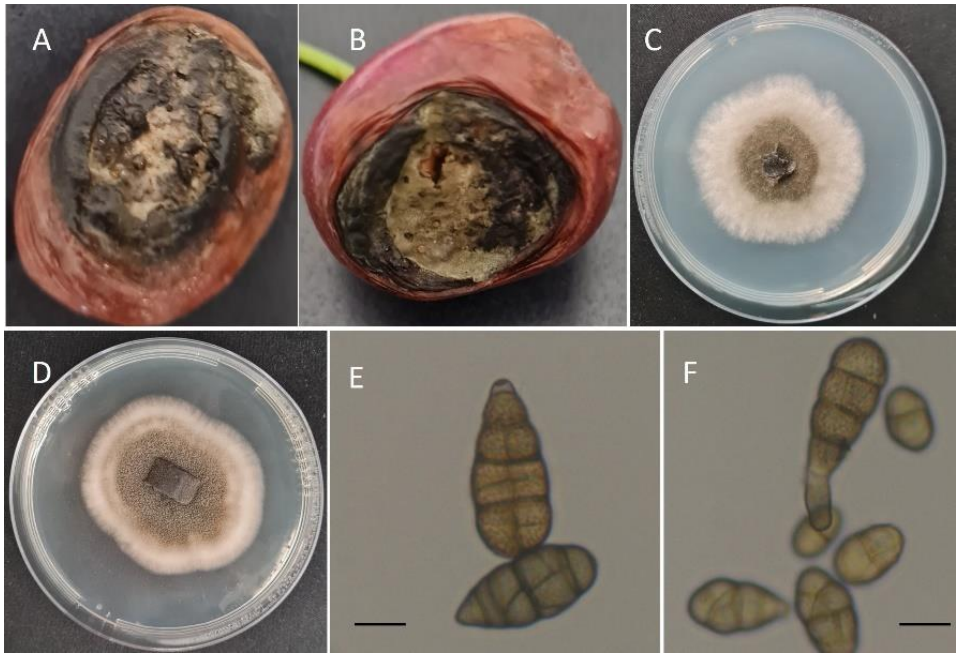


Figure 1. (**A**, **B**) Symptoms of *Alternaria* black rot on naturally infected sweet cherry cv. Regina; (**C**) colony growth of AASC (strain T8) after 7 days on PDA; (**D**) colony growth of *A. alternata* (strain GR13) after 7 days on PDA; (**E**) conidia of AASC (strain T8) obtained after 15 days on PCA; (**F**) conidia of *A. alternata* (strain GR13) obtained after 15 days on PCA. Scale bar: (**E**, **F**) = 10 µm

Table 1. Strain name, species identification, year of isolation, colony growth (cm), size of conidia (length-width) and mean rot diameter obtained with the pathogenicity test for the strains isolated from sweet cherry fruits.

Isolate Name	Fungal Species	Year of Isolation	Cherry Cultivar	Colony Growth (cm ± SD)	Size of Conidia (Length × Width; µm)	Rot Diameter (mm ± SD) [1]
D4	AASC	2020	Kordia	4.94 ± 0.59	21.73 × 11.31	12.06 $^{b-d}$ ± 1.52
T2	AASC	2020	Ferrovia	5.14 ± 0.28	20.13 × 11.19	9.56 ^{bc} ± 1.22
T3	AASC	2020	Ferrovia	4.98 ± 0.55	21.68 × 11.23	12.21 ^{bc} ± 1.66
T6	*A. alternata*	2020	Ferrovia	4.90 ± 0.59	20.64 × 11.18	13.81 $^{b-d}$ ± 1.82
T8	AASC	2020	Ferrovia	4.96 ± 0.55	20.57 × 11.10	10.65 $^{b-d}$ ± 1.86
T9	AASC	2020	Ferrovia	5.08 ± 0.35	21.53 × 11.27	10.75 ^{bc} ± 2.31
GR1	AASC	2020	Regina	4.90 ± 0.58	21.73 × 11.24	11.39 $^{b-d}$ ± 1.88
GR3	*A. alternata*	2020	Regina	4.70 ± 0.51	20.83 × 11.12	9.06 ^{bc} ± 1.81
GR6	AASC	2020	Regina	4.8 8± 0.53	20.89 × 10.99	14.94 $^{b-d}$ ± 2.77
GR8	AASC	2020	Regina	4.94 ± 0.59	20.94 × 11.20	11.94 $^{b-d}$ ± 1.68

GR13	*A. alternata*	2020	Regina	4.82 ± 0.51	20.93 × 11.21	9.06 ^{bc} ± 1.45
W2	AASC	2020	Kordia	5.02 ± 0.33	21.07 × 11.16	11.06 $^{b-d}$ ± 1.53
W6	AASC	2020	Kordia	5.30 ± 0.41	20.43 × 11.12	11.40 $^{b-d}$ ± 2.29
Q1	AASC	2020	Kordia	5.00 ± 0.78	19.76 × 11.64	14.43 $^{b-d}$ ± 2.64
X7	AASC	2020	Kordia	4.96 ± 0.62	20.84 × 11.31	15.25 ^{cd} ± 2.94
Ch1	AASC	2021	Regina	4.70 ± 0.70	21.25 × 11.11	9.38 ^{bc} ± 1.98
Ch2	AASC	2021	Regina	5.16 ± 0.15	20.59 × 10.86	10.94 ^{bc} ± 2.59
Ch4	AASC	2021	Regina	4.94 ± 0.55	21.30 × 11.21	8.43 ^{ab} ± 2.03
Ch5	AASC	2021	Regina	4.76 ± 0.92	21.32 × 11.15	13.93 $^{b-d}$ ± 1.94
Ch11	AASC	2021	Regina	5.12 ± 0.13	20.75 × 11.18	9.11 ^{bc} ± 1.61
Ch12	AASC	2021	Regina	4.96 ± 0.55	20.88 × 11.02	10.71 ^{bc} ± 3.54
Ch13	AASC	2021	Regina	4.94 ± 0.59	21.16 × 11.17	9.12 ^{bc} ± 1.66
Ch14	AASC	2021	Regina	5.26 ± 0.49	20.20 × 10.84	10.06 $^{b-d}$ ± 1.59

Ch16	AASC	2021	Regina	5.12 ± 0.77	21.40 × 11.31	11.75 $^{b-d}$ ± 1.88
Ch17	AASC	2021	Regina	5.08 ± 0.74	21.32 × 11.14	17.00 ^{d} ± 2.13
Ch18	AASC	2021	Regina	4.94 ± 0.47	21.71 × 11.23	14.25 $^{b-d}$ ± 1.89
Ch19	AASC	2021	Regina	4.74 ± 0.81	21.10 × 11.22	12.64 $^{b-d}$ ± 1.61
Ch21	*A. alternata*	2021	Regina	5.06 ± 0.34	21.02 × 10.88	11.00 ^{bc} ± 1.98
Ch22	AASC	2021	Regina	5.00 ± 0.21	20.87 × 11.07	10.06 ^{bc} ± 1.66
Ch23	*A. alternata*	2022	Regina	5.12 ± 0.53	20.90 × 11.03	11.50 $^{b-d}$ ± 1.90
Ch26	AASC	2022	Regina	5.18 ± 0.48	21.04 × 11.12	11.94 $^{b-d}$ ± 1.87
Ch27	AASC	2022	Regina	4.86 ± 0.77	21.12 ×11.09	9.25 ^{bc} ± 1.80
Ch35	AASC	2022	Regina	4.98 ± 0.41	21.03 × 11.16	10.78 $^{b-d}$ ± 1.93
Ch37	*A. alternata*	2022	Regina	4.52 ± 0.78	21.12 × 11.19	9.17 ^{bc} ± 1.52
Ch39	AASC	2022	Regina	4.74 ± 0.57	21.07 × 10.98	11.50 ^{bc} ± 1.93
Ch40	AASC	2022	Regina	5.18 ± 0.26	21.12 × 11.11	10.50 ^{bc} ± 1.74

Ch42	AASC	2022	Regina	5.04 ± 0.27	20.94 × 11.03	12.06 $^{b-d}$ ± 1.52
Ch43	*A. alternata*	2022	Regina	4.68 ± 0.68	21.05 × 11.01	10.88 ^{bc} ± 1.23
Ch45	AASC	2022	Regina	4.92 ± 0.59	21.20 × 11.29	10.81 ^{bc} ± 1.94
Ch48	AASC	2022	Regina	4.88 ± 0.50	21.11 × 11.05	9.33 ^{bc} ± 1.97
Healthy control	-	-	Regina	-	-	0 ^{a}

[1] Values are mean of the rot diameter on nine cherry fruits. Values with the same letter are not different according to Tukey's test ($p \leq 0.05$).

2.2. Micro and Macro-Morphological Characteristics

Alternaria isolates were plated on PDA medium (in triplicate) and incubated at 25 ± 1 °C for the macro-morphological characteristics (color, margin, diameter, and texture), according to Simmons et al. [27]. Mean radial growth was measured after 7 days of incubation (Table 1). For the microscopic features (conidia and conidiophore) the isolates were grown onto Potato Carrot Agar (PCA, HiMedia Laboratories, Mumbai, India) under 12 h light and 12 h dark cycle for 15 days [27]. Thirty conidia per isolate were examined using an Eclipse 55i microscope (Nikon, Tokyo, Japan) at 40× magnification.

2.3. DNA Extraction and PCR Amplification

Genomic DNA of 40 *Alternaria* isolates was extracted with an E.Z.N.A. Fungal DNA mini kit (Omega Bio-tek, Darmstadt, Germany) from 0.1 g mycelium of 7-day-old culture grown on PDA (VWR international) according to the manufacturer's instructions. The quality and concentration of extracted DNA was determined using NanoDrop 2000 spectrophotometer (Thermo scientific, Wilmington, DE, USA). The primers Alt-for and Alt-rev [38] were used to amplify part of Alternaria major allergen gene (Alt-a1). The partial *endopolygalacturonase* gene (endoPG) was amplified using PG3 and PG2b primers [39,40]. The primer sets RPB2-5f2 and fRPB2-7cr [41,42] were used to amplify the part of RNA polymerase second largest subunit (*rpb2*). The primers OPA 10-2R and OPA 10-2L [28] were used to amplify part of an anonymous gene region (OPA10-2). The amplification of all four loci were performed according to PCR amplified conditions described by Prencipe et al. [43]. The PCR cycling conditions adopted for *rpb2* and Alt-a1 were described by Woudenberg et al. [44], and for endoPG and OPA 10-2, by Andrew et al. [28]. The amplification products were analyzed on 1% agarose (VWR International, Milan, Italy) after staining with GelRedTM. PCR products were purified with the PCR Purification Kit (QIAquick®, Hilden, Germany) following manufacturer

instructions, before sequencing by Macrogen Europe B. V. (Amsterdam, The Netherlands).

2.4. Phylogenetic Analysis

Phylogenetic analysis was performed using sequences generated in this study and reference sequences of *Alternaria* spp. [32] (Table S1). After cutting the trimmed regions in Geneious v. 11.1.5 program (Auckland, New Zealand) and manual correction in MEGA v. 7, a dataset 2229 bp of 281 bp for Alt-a1, 479 bp for endoPG, 634 bp for OPA10-2, and 835 bp for *rpb2* was obtained. The sequences were aligned using CLUSTALW in MEGA v. 7 [45]. Phylogenetic analysis was performed using the concatenated dataset (*rpb2*, OPA 10-2, Alt-a1 and endoPG) for the identification of *Alternaria* isolates at species level. The phylogeny was based on maximum parsimony (MP) and Bayesian inference (BI) used in a concatenated analysis. For BI, the best fit evolutionary model for each partitioned locus was estimated using MrModeltest v. 2.3 [46] and incorporated into the analysis. MrBayes v. 3.2.5 [47] 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 at 0.2 and trees were sampled every 1000 generations. The analysis stopped when the average standard deviation of split frequencies was below 0.01. The MP analysis was performed using Phylogenetic Analysis using Parsimony (PAUP) v. 4.0b10 [48]. 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’, with all characters equally weighted 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 bootstrap analysis [49] was based on 1000 replications. Sequences generated in this study were deposited in GenBank (Table S1).

2.5. Pathogenicity Assay

The same isolates used for molecular analysis were used for the pathogenicity test. Conidia of tested *Alternaria* isolates, produced on 21-day-old PDA cultures incubated at 22 ± 1 °C under a 12 h photoperiod, were used to obtain conidial suspensions, which were prepared by scrapping off the conidia from the surface of PDA plates with sterile water and 10 µL of Tween 20, which were then filtered through four layers of sterile gauze, as described by Prencipe et al. [43]. The concentration of the collected spore suspension was adjusted to 10^5 conidia/mL. Pathogenicity tests were conducted on healthy fruits of cherries 'Regina' using the method described by Ahmad et al. [8]. Fruit surfaces were disinfected with 1% sodium hypochlorite for 1 min, washed with distilled water and air dried for 5 min. The experiment was conducted using ten fruits per isolate. Each fruit was inoculated with 1 µL of conidial suspension by creating one wound (1 mm^2) with a sterile needle. Control fruits were treated with sterilized distilled water. Inoculated fruits were placed in plastic trays and covered with a plastic film and incubated at 20 ± 2 °C until symptoms appeared. After 10 days of inoculation, rot diameters were measured. Re-isolations were conducted from the inoculated fruits as described above. Each isolate was tested twice.

2.6. Statistical Analysis

The statistical analysis was performed using SPSS software (IBM SPSS Statistics v. 28.0.1.0). Rot diameters obtained in the pathogenicity test were subjected to the analysis of variance (ANOVA) and the mean values were separated by Tukey test ($p \leq 0.05$).

3. Results

3.1. Fungal Isolation, Identification, and Morphological Characterization

Typical symptoms of dark brown to black patches and sunken lesions were observed on sweet cherry fruits. Out of 180 isolates obtained from symptomatic sweet cherry fruits, 40 isolates were identified as *Alternaria* spp. based on micro and macro-morphological observations [27] (Table 1).

Isolates of *Alternaria* spp. isolated from infected sweet cherry fruits were identified based on their morphological characteristics [27]. All the *Alternaria* isolates formed aerial mycelium on PDA, and most of the colonies were dark greyish in the center with white margins after 7 days of incubation at 25 ± 1 °C under 12 h light and dark cycle. The mycelium becomes dark brown after 10 to 14 days. The mean radial growth of colony was 4.96 ± 0.52 cm (Table 1, Figure 1 C, D). The conidiophores were light brown and the conidia were ellipsoidal or ovoid with 1–4 transverse septa, a mean of 21.01 ± 0.39 µm in length and 11.11 ± 0.26 µm in width (Table 1, Figure 1 E, F).

3.2. Phylogenetic Analysis

The sequences obtained in this study were subjected to a BLAST search in NCBI's GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 10 January 2023)) nucleotide database for preliminary identification. A multilocus phylogenetic analysis was conducted using forty isolates of *Alternaria* spp. based on the sequences from four genes (*rpb2*, OPA 10-2, Alt-a1 and endoPG) and sixty-two reference sequences [32], including the outgroup *Alternaria nobilis*. A total of 184 nucleotides were parsimony-informative, 276 were variable and parsimony-uninformative, and 1769 were constant. A maximum of 1000 equally maximum parsimony (MP) trees were saved (Tree length = 771, CI = 0.642, RI = 0.864 and RC = 0.555). Bootstrap support values from the MP analysis are incorporated on the

Bayesian tree in Figure 2. For the Bayesian analyses (BI), MrModeltest suggested that all partitions should be analyzed with Dirichlet state frequency distributions. The following models were recommended by MrModeltest and used: K80 for Alt-a1, SYM + G for endoPG and *rpb2*, and K80 + I + G for OPA 10-2. In the BI, the Alt-a1 partition had 80 unique site patterns, the endoPG partition had 92 unique site patterns, the OPA 10-2 partition had 136 unique site patterns, the *rpb2* partition had 155 unique site patterns and the analysis ran for 6,595,000 generations, resulting in 6596 trees, of which 4947 trees were samples used to calculate the posterior probabilities. Based on multilocus phylogenetic analysis, thirty-three out of forty strains clustered with AASC, whereas the remaining seven strains belonged to *A. alternata* (Figure 2 and Table 1).

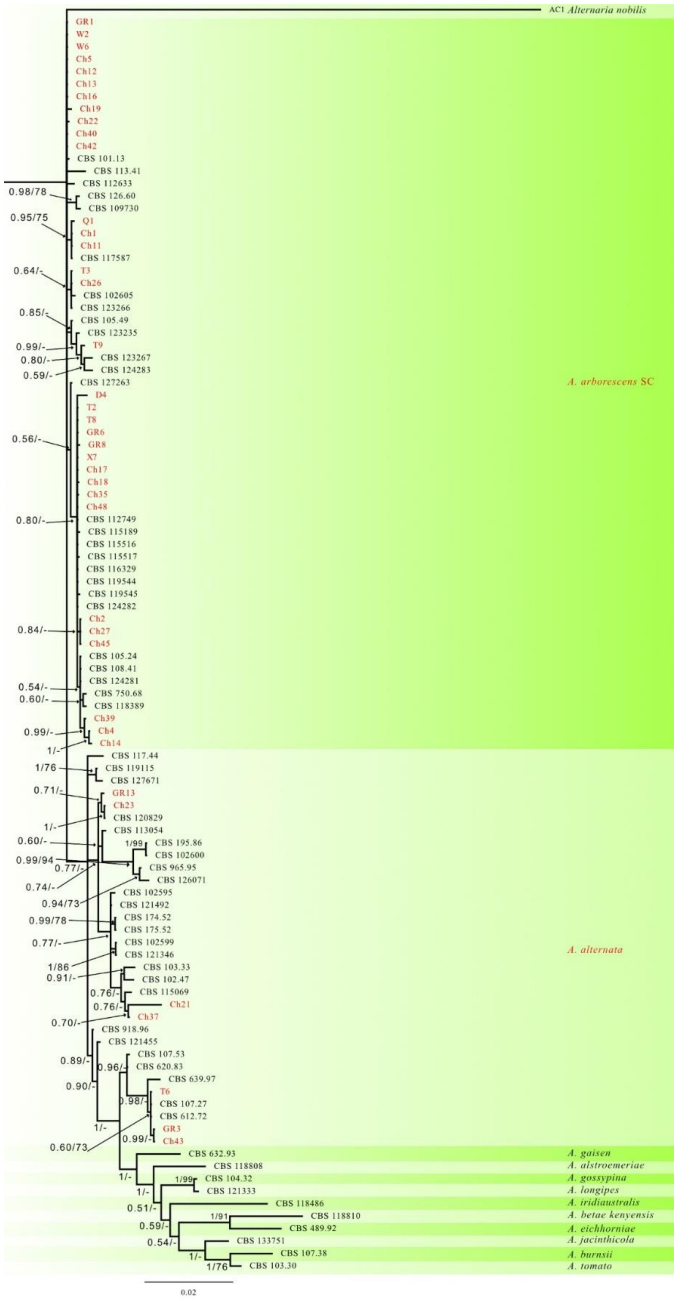


Figure 2. Consensus phylogram of 4947 trees resulting from a Bayesian analysis of the combined *rpb2*, Alta-1, endoPG and OPA 10-2 sequence alignments of the *Alternaria* species. Bootstrap support values and Bayesian posterior probability values are indicated at the nodes. The isolates obtained in this study are in red. The tree was rooted with *Alternaria nobilis* AC1.

3.3. Pathogenicity Assay

The tested *Alternaria* strains were pathogenic on artificially inoculated sweet cherries. Symptoms appeared as the development of black rotted spots and sunken lesions all over the inoculation point after 7 days post inoculation (Figure 3). Lesions increased in diameter with disease progress and morphologically appeared similar to those observed in naturally infected fruits (Figure 3). In our pathogenicity, no significant differences were observed among the *Alternaria* strain/species tested combinations. Only two strains (Ch4 and Ch17) from AASC showed significant differences: the isolate Ch-17 showed the highest rot diameter (17.0 ± 2.13 mm), whereas the isolate Ch-4 showed the lowest rot diameter (8.43 ± 2.03 mm) (Table 1). To fulfill Koch's postulates, re-isolation was carried out on all the symptomatic inoculated fruits, and isolates were identified as *Alternaria* spp. using the *rpb2* gene [41]. Healthy control fruits did not develop any symptoms.

Figure 3. Symptoms of *Alternaria* fruit rot on sweet cherries after 10 days post inoculation (**A**) AASC (strain X7); (**B**) *A. alternata* (strain Ch23); (**C**) healthy control.

4. Discussion

In our study, isolates of *Alternaria* spp. associated with black rot of sweet cherries in Italy were identified as *A. alternata* and *A. arborescens* species complex (AASC) based on morphological and phylogenetic analysis at species level. During three years of investigation, all orchards showed the presence of *A. alternata* and AASC. In the present study, AASC was the predominant fungus isolated from rotted cherries. Along with *Alternaria* spp., we found the co-occurrence of other secondary fungal pathogens associated with rotten fruits, including *Cladosporium* spp., *Monilinia* spp., *Penicillium* spp. and *Botrytis cinerea*. In several studies, AASC has been reported as a causal agent of black rot of mandarin [50], blueberry [51], pomegranate fruit [52], and Japanese plum [53]; core rot of apple [54], leaf blotch and fruit spot diseases of apple [55]; black spots on fruit and leaves of pear [40]; and brown spots on sweet orange and lemon in Italy [56]. No information is reported about the association of AASC with sweet cherries. In contrast, *A. alternata* has been reported as being associated with postharvest rot of sweet cherry [15], black spot of cherry fruits in China [10], and leaf spot disease of sweet cherry in Greece [25] and Turkey [24]. Furthermore, *A. alternata* has been reported as the main causal agent of black rot of cherry tomatoes [57], and fruit rot on strawberry [51,58], mandarin [50], pomegranate fruit [52,59], Japanese plum [53], *Solanum muricatum* [60], and opium poppy [61]. Most studies did not report *Alternaria* spp. as a main pathogen, but in our study, we isolated *Alternaria* spp. as a major pathogen from sweet cherry fruits. In our study, all isolates of *Alternaria* spp. were identified based on cultural and morphological characteristics, as they all formed conidia, similar to the observations of Prencipe et al. [43] and Şimşek et al. [24]. Our *Alternaria* isolates were dark grayish with white margins and formed aerial mycelium on PDA, with an average size of conidia (19.7–21.7 × 10.8–11.6) on PCA. These morphological and cultural characteristics were similar to previous studies [40,62], but different from those described by Şimşek et al. [24]. This phenomenon could be

due to the morphological plasticity of *Alternaria* spp. [17] and the fact that the morphology of conidia is dependent on conidial age and culture conditions [37].

The taxonomy of small-spored *Alternaria* spp. suffered from controversies because *Alternaria* spp. shared similar morphological characteristics and the size of conidia [33,50,63]. Molecular-based assays could be used for the correct identification of *Alternaria* spp. along with morphological characteristics [32,33,64]. Molecular analysis also had some challenges to overcome, as *Alternaria* section *Alternaria* cannot be recognized using standard genetic loci due to the little or no variation in molecular markers [13,28,36,43]. Previous studies suggested that the identification criteria for low resolution of species delimitation in small-spored *Alternaria* spp. are only significant when employing the combination of different genes together [14,17,27]. To overcome the issues, the phylogeny of *Alternaria* sections was solved by using nine gene regions (Alt a 1, endoPG, gapdh, ITS, LSU, OPA10-2, *rpb2*, SSU and tef1) by Woudenberg et al. [32]. The concatenated session of six gene regions of Alt a 1, endoPG, ITS, OPA10-2, *rpb2*, and tef1 was able to separate AASC from *A. alternata* [14]. In our study, we excluded the slowly evolving gene tef 1 and used the genes proposed by Prencipe et al. [43]. Moreover, previous studies confirmed that alt a1 [65] and OPA10-2 [43] were sufficient to separate the *A. alternata* from AASC. The four loci (Alt-a1, endoPG, opa10-2 and *rpb2*) used for the phylogenetic analysis performed in this research allowed us to identify thirty-three isolates as members of AASC, and seven isolates as *A. alternata*. The combined analysis of phylogenetic trees showed similarity with previous studies [14,43]. However, the phylogeny of our study obtained from the concatenated session of four genes has low bootstrap value in agreement with previous studies [35,53]. Considering our phylogenetic analysis results, the inclusion of more genes, such as gaphd, LSU and SSU, could increase the discrimination power, as previously proposed by Zhang et al. [17].

In our pathogenicity test, sweet cherry fruits were wounded and inoculated with conidial suspension of *Alternaria* strains. Pathogenicity results showed that all the *Alternaria* strains were pathogenic, had high virulence, and produced irregular lesions when fruits were inoculated by conidial suspension. According to previous studies, *A. alternata* species were pathogenic on sweet cherry fruits inoculated with conidial suspension and lesions were observed [8]. Additionally, Prencipe et al. [43] reported that isolates of *A. alternata* and AASC were pathogenic when inoculated with conidial suspension on wounded European pear. In previous studies, *Alternaria* spp. was confirmed to be an opportunistic, saprophytic and weak pathogen that enters the plant tissues through natural openings and wounds [24,66,67], when the plant becomes more susceptible to diseases [68]. AASC strains caused lesions ranging from 8.43 to 17.0 mm in size while the lesion size of *A. alternata* strains ranged from 9.06 to 13.81 mm. According to previous studies [55,69], pathogenicity may be isolate-dependent instead of species-dependent. Our results showed that there was little difference among the tested *Alternaria* spp.

In conclusion, the present work describes for the first time the presence of AASC as an agent of black rot on sweet cherry fruits in Italy. Investigation should verify if other species of *Alternaria* could be involved in black rot of cherry fruit. Moreover, more isolates from different geographical areas should be included to explore the genetic diversity of the causal agents of black rot of cherry. Future studies will focus on developing and testing effective disease management strategies both in field and during postharvest. Furthermore, future studies will focus on characterizing the mycotoxin production potential of *Alternaria* species on sweet cherries.

Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jof9100992/s1, Table S1: Collection details

and GenBank accession numbers of strains isolated from sweet cherries in Italy and reference strains included in this study for the phylogenetic analysis.

Author Contributions

Conceptualization, M.W. and D.S.; methodology, M.W., D.S. and S.P.; software, M.W. and V.G.; validation, D.S., V.G. and M.W.; formal analysis, M.W.; investigation, M.W.; resources, D.S.; data curation, M.W.; writing—original draft preparation, M.W.; writing—review and editing, V.G., S.P. and D.S; supervision, D.S.; project administration, D.S.; funding acquisition, D.S. All authors have read and agreed to the published version of the manuscript.

Funding

This work was conducted within the framework of the PRIMA StopMedWaste project, which is funded by PRIMA, a program supported by the European Union.

Institutional Review Board Statement

Not applicable

Informed Consent Statement

Not applicable

Data Availability Statement

Not applicable

Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Materials

Table S1. Collection details and GenBank accession numbers of strains isolated from sweet cherries in Italy and reference strains included in this study for the phylogenetic analysis.

Species	Isolate	Location	Host	*rpb2*	Alt a 1	endoPG	OPA10-2
Alternaria alstroemeriae	CBS 118808; E.G.S. 50.116	USA	*Alstroemeria* sp.	KP124764	KP123845	KP123993	KP124601
A. alternata	CBS 107.27; ATCC 24463; QM 1736 (*A. citri*)	USA	*Citrus limonium*	KP124768	KP123849	KP123996	KP124604
A. alternata	CBS 103.33; E.G.S. 35.182; IHEM 3319 (*A. soliaegyptiaca*)	Egypt	soil	KP124770	KP123852	KP123999	KP124607
A. alternata	CBS 117.44; E.G.S. 06.190; VKM F-1870 (*A. godetiae*)	Denmark	*Godetia* sp.	KP124772	KP123854	KP124001	KP124609
A. alternata	CBS 102.47; E.G.S. 02.062 (*A. citri*)	USA	*Citrus sinensis*	KP124773	KP123855	KP124002	KP124610
A. alternata	CBS 174.52; E.G.S. 39.1613; IMI 068086; QM 1278	USA	*Anemone occidentalis*	DQ677964	KP123856	KP124003	KP124611
A. alternata	CBS 175.52; E.G.S. 35.1619; IMI 068085; QM 1277	USA	*Juncus mertensianus*	KC584445	KP123857	KP124004	KP124612
A. alternata	CBS 107.53; DSM 3187; IFO 5778 (*A. kikuchiana*)	Japan	*Pyrus pyrifolia*	KP124774	KP123858	KP124005	KP124613
A. alternata	CBS 612.72; DSM 62012 (*A. cinerariae*)	Germany	*Senecio cineraria*	KP124777	KP123861	KP124008	KP124615
A. alternata	CBS 620.83; ATCC 15052 (*A. tenuissima*)	USA	*Nicotiana tabacum*	KP124783	KP123868	KP124015	KP124622
A. alternata	CBS 195.86; E.G.S. 36.172; DAOM 185214 (A. angustiovoidea)	Canada	*Euphorbia esula*	KP124785	JQ646398	KP124017	KP124624
A. alternata	CBS 965.95; IMI 289679 (*A. tenuissima*)	India	*Triticum* sp.	KP124791	KP123872	KP124023	KP124629
A. alternata	CBS 918.96; E.G.S. 34.015; IMI 255532 (*A. tenuissima*)	UK	*Dianthus chinensis*	KC584435	AY563302	KP124026	KP124633
A. alternata	CBS 639.97; IMI 366417	Greece	*Helianthus annuus*	KP124795	KP123876	KP124028	KP124635
A. alternata	CBS 102595; E.G.S. 45.100 (*A. limoniasperae*)	USA	*Citrus jambhiri*	KC584408	AY563306	KP124029	KP124636
A. alternata	CBS 102599; E.G.S. 44.166 (*A. turkisafria*)	Turkey	*Minneola tangelo*	KP124798	KP123879	KP124032	KP124639
A. alternata	CBS 102600; E.G.S. 39.181; ATCC 38963	USA	*Citrus reticulata*	KP124799	KP123880	KP124033	KP124640
A. alternata	CBS 113054; CPC 4263 (*A. tenuissima*)	South Africa	*Malus domestica*	KP124814	KP123894	KP124047	KP124656

A. alternata	CBS 115069; CPC 4254 (*A. tenuissima*)	South Africa	*Malus domestica*	KP124815	KP123895	KP124048	KP124657
A. alternata	CBS 119115	Greece	*Prunus* sp.	KP124828	KP123909	KP124062	np*
A. alternata	CBS 120829	Greece	*Punica granatum*	KP124832	KP123912	KP124066	KP124675
A. alternata	CBS 121346; E.G.S. 45.056 (*A. turkisafria*)	South Africa	*Minneola tangelo*	KP124835	KP123914	KP124069	KP124678
A. alternata	CBS 121455; E.G.S. 50.078 (*A. broussonetiae*)	China	*Broussonetia papyrifera*	KP124838	KP123916	KP124072	KP124681
A. alternata	CBS 121492; HSAUP0207 (*Ulocladium cucumisis*)	China	*Cucumis melo*	KP124840	KP123918	KP124074	KP124683
A. alternata	CBS 126071 (*A. tenuissima*)	Namibia	soil	KP124846	KP123924	KP124080	KP124689
A. alternata	CBS 127671; E.G.S. 52.121 (*A. seleniiphila*)	USA	*Stanleya pinnata*	KP124851	KP123929	KP124085	KP124694
A. alternata	T6	Italy	*Prunus avium*	*OP899743*	*OP899663*	*OP899703*	OQ607083
A. alternata	GR3	Italy	*P. avium*	*OP899747*	*OP899667*	*OP899707*	OQ607084
A. alternata	GR13	Italy	*P. avium*	OP899750	OP899670	OP899710	OQ607085
A. alternata	Ch21	Italy	*P. avium*	*OP899767*	*OP899687*	*OP899727*	OQ607086
A. alternata	Ch23	Italy	*P. avium*	*OP899769*	*OP899689*	*OP899729*	OQ607087
A. alternata	Ch37	Italy	*P. avium*	*OP899773*	*OP899693*	*OP899733*	OQ607088
A. alternata	Ch43	Italy	*P. avium*	*OP899777*	*OP899697*	*OP899737*	OQ607089
A. arborescens SC	CBS 101.13; E.G.S. 07.022; QM1765 (*A. geophila*)	Switzerland	peat soil	KP124862	KP123940	KP124096	KP124705
A. arborescens SC	CBS 105.24; IHEM 3123 (*A. alternata*)	Unknown	*Solanum tuberosum*	KP124863	KP123941	KP124097	KP124706
A. arborescens SC	CBS 108.41; E.G.S. 44.087; ATCC 11892 (*A. alternata*)	Unknown	wood	KP124864	KP123942	KP124098	KP124707
A. arborescens SC	CBS 113.41; IHEM 3318 (*A. alternata*)	Unknown	*Schizanthus* sp.	KP124865	KP123943	KP124099	KP124708
A. arborescens SC	CBS 105.49 (*A. alternata*)	Italy	contaminant blood culture	KP124866	KP123944	KP124100	KP124709
A. arborescens SC	CBS 126.60; IMI 081622 (*A. maritima*)	UK	wood	KP124867	JQ646390	KP124101	KP124710
A. arborescens SC	CBS 750.68; LCP 68.1989 (*A. tenuissima*)	France	*Phaseolus vulgaris*	KP124868	KP123945	KP124102	KP124711
A. arborescens SC	CBS 102605; E.G.S. 39.128 (*A. arborescens*)	USA	*Solanum lycopersicum*	KC584377	AY563303	AY295028	KP124712
A. arborescens SC	CBS 109730 (*A. arborescens*)	USA	*Solanum lycopersicum*	KP124869	KP123946	KP124103	KP124713

A. arborescens SC	CBS 112633; CPC 4244 (*A. arborescens*)	South Africa	*Malus domestica*	KP124870	KP123947	KP124104	KP124714
A. arborescens SC	CBS 112749; CPC 4245 (*A. arborescens*)	South Africa	*Malus domestica*	KP124871	KP123948	KP124105	KP124715
A. arborescens SC	CBS 115189; CPC 4345 (*A. arborescens*)	South Africa	*Citrus clementina*	KP124872	KP123949	KP124106	KP124716
A. arborescens SC	CBS 115516; CPC 4247 (*A. arborescens*)	South Africa	*Malus domestica*	KP124873	KP123950	KP124107	KP124717
A. arborescens SC	CBS 115517; CPC 4246 (*A. arborescens*)	South Africa	*Malus domestica*	KP124874	KP123951	KP124108	KP124718
A. arborescens SC	CBS 116329 (*A. alternata*)	Germany	*Malus domestica*	KP124875	KP123952	KP124109	KP124719
A. arborescens SC	CBS 117587 (*A. alternata*)	Netherlands	*Brassica* sp.	KP124876	KP123953	KP124110	KP124720
A. arborescens SC	CBS 118389; E.G.S. 90.131 (*A. gaisen*)	Japan	*Pyrus pyrifolia*	KP124877	KP123954	KP124111	KP124721
A. arborescens SC	CBS 119544; E.G.S. 43.072 (*A. cerealis*)	New Zealand	*Avena sativa*	KP124878	KP123955	KP124112	KP124722
A. arborescens SC	CBS 119545; E.G.S. 48.130 (*A. senecionicola*)	New Zealand	*Senecio skirrhodon*	KP124879	KP123956	KP124113	KP124723
A. arborescens SC	CBS 123235 (*A. alternata*)	Denmark	human toenail	KP124880	KP123957	KP124114	KP124724
A. arborescens SC	CBS 123266 (*A. alternata*)	Denmark	human toenail	KP124881	KP123958	KP124115	KP124725
A. arborescens SC	CBS 123267 (*A. alternata*)	Denmark	human nail	KP124882	KP123959	KP124116	KP124726
A. arborescens SC	CBS 124281 (*A. arborescens*)	Denmark	*Triticum* sp.	KP124883	KP123961	KP124118	KP124728
A. arborescens SC	CBS 124282 (*A. arborescens*)	Denmark	*Hordeum vulgare*	KP124884	KP123962	KP124119	KP124729
A. arborescens SC	CBS 124283 (*A. tenuissima*)	Russia	*Oryza* sp.	KP124885	KP123963	KP124120	KP124730
A. arborescens SC	CBS 127263 (*A. alternata*)	Mexico	Human nasal infection	KP124886	KP123964	KP124121	KP124731
A. arborescens SC	D4	Italy	*P. avium*	OP899740	OP899660	OP899700	OQ607090
A. arborescens SC	T2	Italy	*P. avium*	OP899741	OP899661	OP899701	OQ607091
A. arborescens SC	T3	Italy	*P. avium*	OP899742	OP899662	OP899702	OQ607092
A. arborescens SC	T8	Italy	*P. avium*	OP899744	OP899664	OP899704	OQ607093
A. arborescens SC	T9	Italy	*P. avium*	OP899745	OP899665	OP899705	OQ607094
A. arborescens SC	GR1	Italy	*P. avium*	OP899746	OP899666	OP899706	OQ607095
A. arborescens SC	GR6	Italy	*P. avium*	OP899748	OP899668	OP899708	OQ607096
A. arborescens SC	GR8	Italy	*P. avium*	OP899749	OP899669	OP899709	OQ607097
A. arborescens SC	W2	Italy	*P. avium*	OP899751	OP899671	OP899711	OQ607098

A. arborescens SC	W6	Italy	*P. avium*	OP899752	OP899672	OP899712	OQ607099
A. arborescens SC	Q1	Italy	*P. avium*	OP899753	OP899673	OP899713	OQ607100
A. arborescens SC	X7	Italy	*P. avium*	OP899754	OP899674	OP899714	OQ607101
A. arborescens SC	Ch1	Italy	*P. avium*	OP899755	OP899675	OP899715	OQ607102
A. arborescens SC	Ch2	Italy	*P. avium*	OP899756	OP899676	OP899716	OQ607103
A. arborescens SC	Ch4	Italy	*P. avium*	OP899757	OP899677	OP899717	OQ607104
A. arborescens SC	Ch5	Italy	*P. avium*	OP899758	OP899678	OP899718	OQ607105
A. arborescens SC	Ch11	Italy	*P. avium*	OP899759	OP899679	OP899719	OQ607106
A. arborescens SC	Ch12	Italy	*P. avium*	OP899760	OP899680	OP899720	OQ607107
A. arborescens SC	Ch13	Italy	*P. avium*	OP899761	OP899681	OP899721	OQ607108
A. arborescens SC	Ch14	Italy	*P. avium*	OP899762	OP899682	OP899722	OQ607109
A. arborescens SC	Ch16	Italy	*P. avium*	OP899763	OP899683	OP899723	OQ607110
A. arborescens SC	Ch17	Italy	*P. avium*	OP899764	OP899684	OP899724	OQ607111
A. arborescens SC	Ch18	Italy	*P. avium*	OP899765	OP899685	OP899725	OQ607112
A. arborescens SC	Ch19	Italy	*P. avium*	OP899766	OP899686	OP899726	OQ607113
A. arborescens SC	Ch22	Italy	*P. avium*	OP899768	OP899688	OP899728	OQ607114
A. arborescens SC	Ch26	Italy	*P. avium*	OP899770	OP899690	OP899730	OQ607115
A. arborescens SC	Ch27	Italy	*P. avium*	OP899771	OP899691	OP899731	OQ607116
A. arborescens SC	Ch35	Italy	*P. avium*	OP899772	OP899692	OP899732	OQ607117
A. arborescens SC	Ch39	Italy	*P. avium*	OP899774	OP899694	OP899734	OQ607118
A. arborescens SC	Ch40	Italy	*P. avium*	OP899775	OP899695	OP899735	OQ607119
A. arborescens SC	Ch42	Italy	*P. avium*	OP899776	OP899696	OP899736	OQ607120
A. arborescens SC	Ch45	Italy	*P. avium*	OP899778	OP899698	OP899738	OQ607121
A. arborescens SC	Ch48	Italy	*P. avium*	OP899779	OP899699	OP899739	OQ607122
Alternaria betae-kenyensis	CBS 118810; E.G.S. 49.159; IMI 385709	Kenya	Beta vulgaris var. cicla	KP124888	KP123966	KP124123	KP124733
Alternaria burnsii	CBS 107.38; E.G.S. 06.185	India	*Cuminum cyminum*	KP124889	KP123967	KP124124	KP124734

Alternaria eichhorniae	CBS 489.92; ATCC 22255; ATCC 46777; IMI 121518	India	*Eichhornia crassipes*	KP124895	KP123973	KP124130	KP124740
Alternaria gaisen	CBS 632.93; E.G.S. 90.512	Japan	*Pyrus pyrifolia*	KC584399	KP123974	AY295033	KP124742
Alternaria gossypina	CBS 104.32	Zimbabwe	*Gossypium* sp.	KP124900	JQ646395	KP124135	KP124746
Alternaria iridiaustralis	CBS 118486; E.G.S. 43.014	Australia	*Iris* sp.	KP124905	KP123981	KP124140	KP124751
Alternaria jacinthicola	CBS 133751; MUCL 53159	Mali	*Eichhornia crassipes*	KP124908	KP123984	KP124143	KP124754
Alternaria longipes	CBS 121333; E.G.S. 30.051	USA	*Nicotiana tabacum*	KP124914	KP123990	KP124150	KP124761
Alternaria tomato	CBS 103.30	Unknown	*Solanum lycopersicum*	KP124915	KP123991	KP124151	KP124762
Alternaria nobilis	AC1	Japan	*Dianthus barbatus*	LC476798	LC481624	LC480952	np

*np: no product

References

1. FAOSTAT. https://www.fao.org/faostat/en/#data/QCL/visualize (accessed on 27 February 2023).

2. ISTAT. http://dati.istat.it/Index.aspx?QueryId=33705&lang=en (accessed on 14 March 2023).

3. Padilla-Zakour, O.I.; Ryona, I.; Cooley, H.J.; Robinson, T.L.; Osborne, J.; Freer, J. Shelf-Life Extension of Sweet Cherries by Field Management, Post-Harvest Treatments and Modified Atmosphere Packaging. *N. Y. Fruit. Q.* **2007**, *15*, 3–6.

4. Chiabrando, V.; Garavaglia, L.; Giacalone, G. The Postharvest Quality of Fresh Sweet Cherries and Strawberries with an Active Packaging System. *Foods* **2019**, *8*, 335.

5. Sehirli, S.; Karabulut, O.A.; Ilhan, K.; Sehirli, A. Use and Efficiency of Disinfectants within a Hydrocooler System for Postharvest Disease Control in Sweet Cherry. *Int. J. Fruit. Sci.* **2020**, *20*, S1590–S1606.

6. Maghenzani, M.; Chiabrando, V.; Santoro, K.; Spadaro, D.; Giacalone, G. Essential oil vapour treatment (*Thymus vulgaris* and *Satureja montana*) on postharvest quality of sweet cherry (cv Ferrovia). *J. Food Nutr. Res.* **2018**, *57*, 161–169.

7. Mari, M.; Spadaro, D.; Casals, C.; Collina, M.; De Cal, A.; Usall, J. Postharvest Diseases of Stone Fruits. In *Postharvest Pathology of Fresh Horticultural Produce*; Palou, L., Smilanick, J.L., Eds.; CRC Press: Boca Raton, FL, USA, 2019; pp. 111–140, ISBN 9781138630833.

8. Ahmad, T.; Liu, Y.; Shujian, H.; Moosa, A. First Record of *Alternaria alternata* Causing Postharvest Fruit Rot of Sweet Cherry (*Prunus avium*) in China. *Plant Dis.* **2020**, *104*, 2030.

9. Cancino, S.; Lolas, M.A.; Galdós, L.; Hernández, Y.; Ferrada, E.; Riveros, P.; Blanco-Ulate, B.; Díaz, G.A. Occurrence of *Alternaria alternata* and *A. tenuissima* Causing Black Rot in Cherry Fruits (*Prunus avium*) in Central Chile. *Plant Dis.* **2023**.

10. Zhao, Y.Z.; Liu, Z.H. First Report of Black Spot Disease Caused by *Alternaria alternata* on Cherry Fruits in China. *Plant Dis.* **2012**, *96*, 1580.
11. Latinovi´c, N.; Radišek, S.; Latinovi´c, J. First Report of *Alternaria alternata* Causing Fruit Rot on Fig (*Ficus carica*) in Montenegro. *Plant Dis.* **2014**, *98*, 424.
12. Xiang, M.L.; Li, S.C.; Wu, F.; Zhao, X.Y.; Wang, Y.B.; An, X.X.; Zhang, Y.N.; Chen, M. First Report of *Alternaria alternata* Causing Fruit Rot on *Tetradium ruticarpum* in China. *Plant Dis.* **2021**, *105*, 1194.
13. Lawrence, D.P.; Gannibal, P.B.; Peever, T.L.; Pryor, B.M. The Sections of *Alternaria*: Formalizing Species-Group Concepts. *Mycologia* **2013**, *105*, 530–546.
14. Mati´c, S.; Tabone, G.; Garibaldi, A.; Gullino, M.L. Alternaria Leaf Spot Caused by *Alternaria* Species: An Emerging Problem on Ornamental Plants in Italy. *Plant Dis.* **2020**, *104*, 2275–2287.
15. Thomma, B.P.H.J. AlternariasSpp.: From General Saprophyte to Specific Parasite. *Mol. Plant Pathol.* **2003**, *4*, 225–236.
16. Woudenberg, J.H.C.; Groenewald, J.Z.; Binder, M.; Crous, P.W. *Alternaria* Redefined. *Stud. Mycol.* **2013**, *75*, 171–212.
17. Zhang, M.-J.; Zheng, X.-R.; Li, H.; Chen, F.-M. Alternaria alternata, the Causal Agent of a New Needle Blight Disease on Pinus Bungeana. *J. Fungi* **2023**, *9*, 71.
18. Abata, L.K.; Paz, I.A.; Viera, W.; Flores, F.J. First Report of Alternaria Rot Caused by *Alternaria alternata* on Peach in Ecuador. *Plant Dis.* **2016**, *100*, 2323.
19. Dogan, A.; Cat, A.; Catal, M.; Erkan, M. First Report of *Alternaria alternata* Causing Postharvest Decay in Fig (*Ficus carica* L. Cv. Bursa Siyahi) Fruit in Turkey. *J. Biotechnol.* **2018**, *280*, S84.
20. Gur, L.; Reuveni, M.; Cohen, Y. Occurrence and Etiology of Alternaria Leaf Blotch and Fruit Spot of Apple Caused by *Alternaria alternata* f. sp. mali on cv. Pink Lady in Israel. *Eur. J. Plant Pathol.* **2017**, *147*, 695–708.
21. Moslemi, A.; Ades, P.K.; Groom, T.; Nicolas, M.E.; Taylor, P.W.J. *Alternaria infectoria* and *Stemphylium herbarum*, Two New Pathogens of Pyrethrum

(*Tanacetum cinerariifolium*) in Australia. *Australas. Plant Pathol.* **2017**, *46*, 91–101.

22. Munhuweyi, K.; Lennox, C.L.; Meitz-Hopkins, J.C.; Caleb, O.J.; Opara, U.L. Major Diseases of Pomegranate (*Punica granatum* L.),
Their Causes and Management—A Review. *Sci. Hortic.* **2016**, *211*, 126–139.

23. Ustun, R.; Cat, A.; Uzun, B.; Catal, M. First Report of Alternaria Alternata Causing Leaf Spot Disease on Soybean (*Glycine max*) in Antalya Province of Turkey. *Plant Dis.* **2019**, *103*, 3284.

24. ¸Sim¸sek, A.; Dinler, H.; Uysal Morca, A. Identification and Pathogenicity of *Alternaria Alternata* Causing Leaf Spot Disease on Sweet Cherry in Province of Turkey. *J. Plant Dis. Prot.* **2022**, *129*, 1355–1366.

25. Thomidis, T.; Tsipouridis, C. First Report of Alternaria Leaf Spot on Cherry Trees in Greece. *Plant Dis.* **2006**, *90*, 680.

26. Van de Perre, E.; Jacxsens, L.; Liu, C.; Devlieghere, F.; De Meulenaer, B. Climate impact on Alternaria moulds and their mycotoxins in fresh produce: The case of the tomato chain. *Food Res. Int.* **2015**, *68*, 41–46.

27. Simmons, E.G. *Alternaria: An Identification Manual*; CBS Fungal Biodiversity Centre: Utrecht, The Netherlands, 2007; ISBN 9070351684.

28. Andrew, M.; Peever, T.L.; Pryor, B.M. An Expanded Multilocus Phylogeny Does Not Resolve Morphological Species within the Small-Spored *Alternaria* Species Complex. *Mycologia* **2009**, *101*, 95–109.

29. Ma, G.; Bao, S.; Zhao, J.; Sui, Y.; Wu, X. Morphological and Molecular Characterization of Alternaria Species Causing Leaf Blight on Watermelon in China. *Plant Dis.* **2021**, *105*, 60–70.

30. Somma, S.; Pose, G.; Pardo, A.; Mulè, G.; Pinto, V.F.; Moretti, A.; Logrieco, A.F. AFLP Variability, Toxin Production, and Pathogenicity of *Alternaria* Species from Argentinean Tomato Fruits and Puree. *Int. J. Food Microbiol.* **2011**, *145*, 414–419.

31. Stewart, J.E.; Andrew, M.; Bao, X.; Chilvers, M.I.; Carris, L.M.; Peever, T.L. Development of Sequence Characterized Amplified Genomic Regions (SCAR) for Fungal Systematics: Proof of Principle Using *Alternaria*, *Ascochyta* and *Tilletia*. *Mycologia* **2013**, *105*, 1077–1086.

32. Woudenberg, J.H.C.; Seidl, M.F.; Groenewald, J.Z.; de Vries, M.; Stielow, J.B.; Thomma, B.P.H.J.; Crous, P.W. *Alternaria* Section *Alternaria*: Species, *Formae speciales* or Pathotypes? *Stud. Mycol.* **2015**, *82*, 1–21.

33. Lawrence, D.P.; Rotondo, F.; Gannibal, P.B. Biodiversity and Taxonomy of the Pleomorphic Genus Alternaria. *Mycol. Prog.* **2016**, *15*, 3.

34. Ozkilinc, H.; Rotondo, F.; Pryor, B.M.; Peever, T.L. Contrasting Species Boundaries between Sections *Alternaria* and Porri of the Genus *Alternaria*. *Plant Pathol.* **2018**, *67*, 303–314.

35. Peever, T.L.; Ibañez, A.; Akimitsu, K.; Timmer, L.W. Worldwide Phylogeography of the Citrus Brown Spot Pathogen, *Alternaria alternata*. *Phytopathology* **2002**, *92*, 794–802.

36. Peever, T.L.; Su, G.; Carpenter-Boggs, L.; Timmer, L.W. Molecular Systematics of Citrus-Associated *Alternaria* Species. *Mycologia* **2004**, *96*, 119.

37. Pryor, B.M.; Michailides, T.J. Morphological, Pathogenic, and Molecular Characterization of *Alternaria* Isolates Associated with Alternaria Late Blight of Pistachio. *Phytopathology* **2002**, *92*, 406–416.

38. Hong, S.G.; Cramer, R.A.; Lawrence, C.B.; Pryor, B.M. Alt a 1 Allergen Homologs from *Alternaria* and Related Taxa: Analysis of Phylogenetic Content and Secondary Structure. *Fungal Genet. Biol.* **2005**, *42*, 119–129.

39. Isshiki, A.; Akimitsu, K.; Yamamoto, M.; Yamamoto, H. Endopolygalacturonase Is Essential for Citrus Black Rot Caused by *Alternaria Citri* but Not Brown Spot Caused by *Alternaria alternata*. *Mol. Plant Microbe Interact.* **2001**, *14*, 749–757.

40. Isshiki, A.; Akimitsu, K.; Nishio, K.; Tsukamoto, M.; Yamamoto, H. Purification and Characterization of an Endopolygalacturonase from the Rough Lemon Pathotype of *Alternaria alternata*, the Cause of Citrus Brown Spot Disease. *Physiol. Mol. Plant Pathol.* **1997**, *51*, 155–167.

41. Sung, G.H.; Sung, J.M.; Hywel-Jones, N.L.; Spatafora, J.W. A Multi-Gene Phylogeny of Clavicipitaceae (Ascomycota, Fungi): Identification of Localized Incongruence Using a Combinational Bootstrap Approach. *Mol. Phylogenet Evol.* **2007**, *44*, 1204–1223.

42. Liu, Y.J.; Whelen, S.; Hall, B.D. Phylogenetic Relationships among Ascomycetes: Evidence from an RNA Polymerse II Subunit. *Mol. Biol. Evol.* **1999**, *16*, 1799–1808.

43. Prencipe, S.; Meloni, G.R.; Nari, L.; Schiavon, G.; Spadaro, D. Pathogenicity, Molecular Characterization and Mycotoxigenic Potential of *Alternaria* Spp. Agents of Black Spots on Fruit and Leaves of Pyrus Communis in Italy. *Phytopathology* **2022**, *113*, 309–320.

44. Woudenberg, J.H.C.; Truter, M.; Groenewald, J.Z.; Crous, P.W. Large-Spored *Alternaria* Pathogens in Section Porri Disentangled. *Stud. Mycol.* **2014**, *79*, 1–47.

45. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874.

46. Nylander, J.A.A. MrModeltest Version 2. Program Distributed by the Author. Evolutionary Biology Centre, Uppsala University, Uppsala. References-Scientific Research Publishing. 2004. Available online: https://www.scirp.org/(S(i43dyn45teexjx455qlt3d2q))/reference/ReferencesPapers.aspx?ReferenceID=1276217 (accessed on 29 March 2023).

47. Ronquist, F.; Teslenko, M.; van der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.A.; Huelsenbeck, J.P. MrBayes 3.2: Efficient

Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. *Syst. Biol.* **2012**, *61*, 539–542.

48. Swofford, D.L. PAUP. Phylogenetic Analysis Using Parsimony (and Other Methods). Version 4. Sinauer Associates, Sunderland. References-Scientific Research Publishing. 2003. Available online: https://www.scirp.org/(S(czeh2tfqyw2orz553k1w0r45))/reference/ReferencesPapers.aspx?ReferenceID=1085917 (accessed on 29 March 2023).

49. Hillis, D.M.; Bull, J.J. An Empirical Test of Bootstrapping as a Method for Assessing Confidence in Phylogenetic Analysis. *Syst. Biol.* **1993**, *42*, 182–192.

50. Wang, F.; Saito, S.; Michailides, T.J.; Xiao, C.L. Phylogenetic, Morphological, and Pathogenic Characterization of *Alternaria* Species Associated with Fruit Rot of Mandarin in California. *Plant Dis.* **2021**, *105*, 1555–1567.

51. Zhu, X.Q.; Xiao, C.L. Phylogenetic, Morphological, and Pathogenic Characterization of *Alternaria* Species Associated with Fruit Rot of Blueberry in California. *Phytopathology* **2015**, *105*, 1555–1567.

52. Kanetis, L.; Testempasis, S.; Goulas, V.; Samuel, S.; Myresiotis, C.; Karaoglanidis, G.S. Identification and Mycotoxigenic Capacity of Fungi Associated with Pre- and Postharvest Fruit Rots of Pomegranates in Greece and Cyprus. *Int. J. Food Microbiol.* **2015**, *208*, 84–92.

53. Riquelme, D.; Zuniga, C.; Tapia, E. First Report of Fruit Rot of Sweet Cultivars of Japanese Plum Caused by *Alternaria alternata*, *A. arborescens*, and *A. tenuissima* in Chile. *Plant Dis.* **2021**, *105*, 4167.

54. Ntasiou, P.; Myresiotis, C.; Konstantinou, S.; Papadopoulou-Mourkidou, E.; Karaoglanidis, G.S. Identification, Characterization and Mycotoxigenic Ability of *Alternaria* spp. Causing Core Rot of Apple Fruit in Greece. *Int. J. Food Microbiol.* **2015**, *197*, 22–29.

55. Harteveld, D.O.C.; Akinsanmi, O.A.; Drenth, A. Multiple *Alternaria* Species Groups Are Associated with Leaf Blotch and Fruit Spot Diseases of Apple in Australia. *Plant Pathol.* **2013**, *62*, 289–297.

56. Aiello, D.; Guarnaccia, V.; Azzaro, A.; Polizzi, G. Alternaria Brown Spot on New Clones of Sweet Orange and Lemon in Italy. *Phytopathol. Mediterr.* **2020**, *59*, 131–145.

57. Yang, J.; Chen, Y.Z.; Yu-Xuan, W.; Tao, L.; Zhang, Y.D.; Wang, S.R.; Zhang, G.C.; Zhang, J. Inhibitory Effects and Mechanisms of Vanillin on Gray Mold and Black Rot of Cherry Tomatoes. *Pestic. Biochem. Physiol.* **2021**, *175*, 104859.

58. Al-Rahbi, B.A.A.; Al-Sadi, A.M.; Al-Mahmooli, I.H.; Al-Maawali, S.S.; Al-Mahruqi, N.M.T.; Velazhahan, R. Meyerozyma guilliermondii SQUCC-33Y Suppresses Postharvest Fruit Rot of Strawberry Caused by *Alternaria Alternata*. *Australas. Plant Pathol.* **2021**, *50*, 349–352.

59. Ezra, D.; Shulhani, R.; Bar Ya'Akov, I.; Harel-Beja, R.; Holland, D.; Shtienberg, D. Factors Affecting the Response of Pomegranate Fruit to *Alternaria alternata*, the Causal Agent of Heart Rot. *Plant Dis.* **2019**, *103*, 315–323.

60. Chen, M.; Jia, M.S.; Li, S.C.; Xiao, L.H.; Wang, Y.B.; Peng, W.W.; Chen, J.Y.; Xiang, M.L. First Report of Postharvest Fruit Rot in *Solanum muricatum* Caused by *Alternaria alternata* in Southwest China. *Plant Dis.* **2022**, *106*, 2520.

61. Guo, L.W.; He, S.H.; Gao, Z.R.; Wu, Z.T.; Duan, R.Q.; Yang, K.Z.; Wei, Y.J.; He, X.H. First Report of Fruit Rot in Opium Poppy (*Papaver somniferum*) Caused by *Alternaria alternata* in China. *Plant Dis.* **2020**, *104*, 3264.

62. Basım, E.; Basım, H.; Abdulai, M.; Baki, D.; Öztürk, N. Identification and Characterization of *Alternaria alternata* Causing Leaf Spot of Olive Tree (*Olea europaea*) in Turkey. *Crop Prot.* **2017**, *92*, 79–88.

63. Serdani, M.; Kang, J.C.; Andersen, B.; Crous, P.W. Characterisation of *Alternaria* Species-Groups Associated with Core Rot of Apples in South Africa. *Mycol. Res.* **2002**, *106*, 561–569.

64. Pryor, B.M.; Gilbertson, R.L. Molecular Phylogenetic Relationships amongst *Alternaria* Species and Related Fungi Based upon Analysis of Nuclear ITS and Mt SSU RDNA Sequences. *Mycol. Res.* **2000**, *104*, 1312–1321.
65. Elfar, K.; Zoffoli, J.P.; Latorre, B.A. Identification and Characterization of *Alternaria* Species Associated with Moldy Core of Apple in Chile. *Plant Dis.* **2018**, *102*, 2158–2169.
66. Prusky, D. Pathogen Quiescence in Postharvest Diseases. *Annu. Rev. Phytopathol.* **1996**, *34*, 413–434.
67. Rotem, J. The Genus Alternaria: Biology, Epidemiology, and Pathogenicity; *The American Phytopathological Society*: Saint Paul, MN, USA, 1994; ISBN 978-0-89054-152-4.
68. Mmbaga, M.T.; Shi, A.; Kim, M.-S. Identification of *Alternaria alternata* as a Causal Agent for Leaf Blight in Syringa Species. *Plant Pathol J* **2011**, *27*, 120–127.
69. Fontaine, K.; Fourrier-Jeandel, C.; Armitage, A.D.; Boutigny, A.L.; Crépet, M.; Caffier, V.; Gnide, D.C.; Shiller, J.; Le Cam, B.; Giraud, M.; et al. Identification and Pathogenicity of *Alternaria* Species Associated with Leaf Blotch Disease and Premature Defoliation in French Apple Orchards. *Peer J* **2021**, *9*, e12496.

Disclaimer/Publisher's Note

Chapter 3

Molecular Characterization and Pathogenicity of *Diaporthe* Species Causing Nut Rot of Hazelnut in Italy

Muhammad Waqas[1], Vladimiro Guarnaccia[1,2], S. Bardella[3], Davide Spadaro[1,2,†]

[1]Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy
[2]AGROINNOVA – Centre of Competence for the Innovation in the Agro-environmental Sector, University of Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy
[3]Fondazione Agrion - Via Falicetto, 24, 12030 Manta (CN), Italy

†Corresponding author: D. Spadaro; davide.spadaro@unito.it

Keywords

Corylus avellana; *Diaporthe eres*; hazelnut; nut rot; pathogenicity; phylogeny

Published in Plant Disease (2024), https://doi.org/10.1094/PDIS-01-23-0168-RE

Abstract

Hazelnut (*Corylus avellana*), a nut crop that is rapidly expanding worldwide, is endangered by a nut rot. Nut rot results in hazelnut defects. A survey was conducted in north-western Italy during 2020 and 2021 to identify the causal agents of hazelnut rots. Typical symptoms of black rot, mold, and necrotic spots were observed on hazelnut nuts. The prevalent fungi isolated from symptomatic hazelnut kernels were *Diaporthe* spp. (38%), *Botryosphaeria dothidea* (26%), *Diplodia seriata* (14%), and other fungal genera with less frequent occurrences. Among 161 isolated *Diaporthe* spp., 40 were selected for further analysis. Based on morphological characterization and multi-locus phylogenetic analysis of the ITS, *tef1-α*, and *tub2*, seven *Diaporthe* species were identified, as *D. eres*, *D. foeniculina*, *D. novem*, *D. oncostoma*, *D. ravennica*, *D. rudis*, and *D. sojae*. *D. eres* was the main species isolated from hazelnut rots, in particular from moldy nuts. Pathogenicity test performed on hazelnut nuts 'Tonda Gentile del Piemonte' using a mycelium plug showed that all the *Diaporthe* isolates were pathogenic on their original host. To our knowledge, this work is the first report of *D. novem*, *D. oncostoma* and *D. ravennica* on hazelnut nuts worldwide. *Diaporthe foeniculina*, *D. rudis*, and *D. sojae* were reported for the first time as agents of hazelnut nut rot in Italy. Future studies should focus on the comprehension of epidemiology and climatic conditions favoring the development of *Diaporthe* spp. on hazelnut. Prevention and control measures should target *D. eres*, representing the main causal agents responsible for defects and nut rot of hazelnuts in Italy.

Introduction

Hazelnut (*Corylus avellana* L.) belongs to Betulaceae family, which is native to Europe and Western Asia, where it is widely distributed (Arciuolo et al. 2020). Hazelnut production increased in the last several years in response to a high demand for its health benefits from fiber and nutrients (Glei et al. 2018; Nunzio 2019), but

also for its massive use in spreadable creams and other confectionery products (Silvestri et al. 2021). The main producers of hazelnuts are Turkey, Italy, and the USA. In Italy, hazelnut is the major nut crop, cultivated on 84,440 ha, with an annual production of about 118,791 t (ISTAT 2022). The cultivation of hazelnut is spread all over Italy, from north to south, with main production regions as Piedmont (13%), Latium (38%) and Campania (37%) (Scarpari et al. 2020).

Hazelnut production is threatened by various fungal pathogens and disorders which reduce nut quality and yield by altering its kernel (Arciuolo et al. 2022; Battilani et al. 2018; Teviotdale et al. 2002) and can release mycotoxins (Spadaro et al. 2020; Valente et al. 2020). Several defects are reported on hazelnut kernels, such as inner discoloration, necrosis, and presence of blemishes, which reduce the market quality standards (Arciuolo et al. 2020). Hazelnut rot, called "avariato" (spoiled) in Italian, affects 3 to 7% of the nuts, every year, causing significant economic losses (Vitale et al. 2020). Different fungal genera were isolated from rotten hazelnuts: *Alternaria*, *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Diaporthe*, *Fusarium*, *Penicillium Pestalotiopsis*, and *Phoma* in the Caucasus region (Battilani et al. 2018); *Alternaria*, *Colletotrichum*, *Fusarium*, and *Phomopsis* in central Italy (Librandi et al. 2006); *Alternaria*, *Botryosphaeria*, *Cladosporium*, *Colletotrichum*, *Diaporthe*, *Didymella*, *Fusarium*, and *Phoma* in northern and southern Italy (Vitale et al. 2020). Among the fungal pathogens, *Diaporthe* spp. Seem to play a major role in the occurrence of hazelnut defects in the Caucasus region (Arciuolo et al. 2022; Battilani et al. 2018), but it is unclear which of the above-mentioned fungal genera play a key role in Italy. The genus *Diaporthe* (anamorph *Phomopsis*) belongs to Diaporthaceae family and was originally established with *Diaporthe eres* as the typified species isolated from *Ulmus* sp. in Germany (Nitschke 1870; Senanayake et al. 2017). The members of *Diaporthe* genus represent a cosmopolitan group of fungi which include plant pathogens, saprobes on decaying tissues and endophytes widely distributed in tropical, and temperate regions worldwide (Guarnaccia et al. 2018; Marin-Felix et

al. 2019; Yang et al. 2020). *Diaporthe* spp. are causal agents of diseases on a wide range of economically important plant hosts, such as horticultural, forest, ornamentals, and fruit crops (Bertetti et al. 2018; Dissanayake et al. 2017; Guarnaccia and Crous, 2018; Huang et al. 2015; Prencipe et al. 2017; Thompson et al. 2011; Udayanga et al. 2014; Yang et al. 2018).

Historically, *Diaporthe* spp. were considered monophyletic group based on unique and typical *Phomopsis* spp. asexual and sexual morphs (Gomes et al. 2013). However, paraphyletic nature was revealed by Gao et al. (2017) showing that the genera *Phaeocytostroma*, *Stenocarpella* (Lamprecht et al. 2011), *Pustulomyces* (Dai et al. 2014), *Ophiodiaporthe* (Fu et al. 2013) and *Mazzantia* (Wehmeyer 1926) are embedded in *Diaporthe s. lat.* Moreover, Senanayake et al. (2017) included *Diaporthe*-like clades within the order Diaporthales.

Pathogen identification at species level is crucial to understand the biology and epidemiology and to develop an appropriate disease management (Santos et al. 2017; Yang et al. 2018). Traditionally, *Diaporthe* species identification was based on culture characteristics, morphology, and host association (Udayanga et al. 2011; Yang et al. 2020), but morphological identification was unreliable for species identification because of high similarity of *Diaporthe* spp. (Dissanayake et al. 2017; Udayanga et al. 2011). Several studies based on the use of multi-locus phylogenetic analyses solved the boundaries within *Diaporthe* genus (Gomes et al. 2013; Marin-Felix et al. 2019; Udayanga et al. 2012). Mostly, internal transcribed spacer (ITS), translation elongation factor-1α (*tef-1α*), beta tubulin (*β-tubulin)*, calmodulin *(CAL)*, and histone *(HIS)* genes are used for molecular characterization of *Diaporthe* spp. (Guarnaccia et al. 2018; Yang et al. 2020).

In Northern Italy, hazelnuts often show nut rot. Several fungi have been associated to nut rot in Italy (Librandi et al. 2006; Vitale et al. 2020). However, the role of the causal agents, and in particular of Diaporthe spp., has not been clarified. Moreover, it is unclear which species of Diaporthe are involved in the development of hazelnut

nut rot. In order to investigate and better understand the etiology of defected kernels, a monitoring of their possible causal agents was performed between 2020 and 2021. The aims of the present study were (i) to isolate and identify the fungal species associated with the hazelnut nuts defects in Northern Italy, (ii) to evaluate the genetic diversity of *Diaporthe* spp. associated with hazelnut nuts defects in Italy, and (iii) to evaluate the pathogenicity of the species found.

Materials and methods

Sampling and isolation

Field surveys were conducted during November-December each in 2020 and 2021 in nine orchards located in Piedmont, Northern Italy. A total of 420 nut samples were collected from defected hazelnuts (moldy, black rotted, and necrotic) at BBCH 89 (nuts separated from the husk at the basal scar; Paradinas et al. 2022) (**Fig. 1**). Symptomatic kernels cut in half were disinfected in 1% sodium hypochlorite for 1 min, rinsed in sterile water for 1 min, and dried on sterile filter paper. Then five small pieces of half cut kernels were placed on potato dextrose agar (PDA, VWR international, Leuven, Belgium) containing streptomycin (0.025g/l). The PDA plates (9 cm diameter) were incubated at 23 ± 2 °C with a cycle of 12 h of light and 12 h of darkness for 2-3 days depending on colony growth. Pure cultures were obtained after 10 days by transferring the mycelium plug from the edge of the colonies and placed in fresh PDA plates. Isolates used in this study were maintained and kept at -80 °C in the culture collection of the University of Turin, Torino, Italy.

Macro- and micro-morphological analysis

Mycelial plugs (5 mm diameter) were taken from the margins of actively growing colonies on PDA and transferred onto the center of Petri dishes containing 2% tap water agar supplemented with sterile pine needles (PNA; Smith et al. 1996) and

PDA and incubated at room temperature (22± 2 °C) under a 12-h near-ultraviolet light/12-h dark cycle to induce sporulation according to Gomes et al. (2013) and Lombard et al. (2014). Colony characters and pigment production on PDA and PNA were noted after 7 and 10 days. Colony colors were ranked according to Rayner (1970). Cultures were observed periodically for the development of ascomata and conidiomata. Morphological characteristics were examined by mounting fungal structures in clear lactic acid. Using an Eclipse 55i microscope (Nikon, Tokyo, Japan), 30 measurements at 1000× magnification were performed per isolate. Initially, identification was performed by colony and conidial morphology as described by Phillips et al. (2013), and then based on molecular identification by using internal transcribed spacer sequence amplified with primers ITS 1 and ITS 4 (White et al. 1990).

DNA extraction and PCR amplification

Forty isolates of *Diaporthe* species were selected based on morphological analysis, DNA sequence, and geographical origin data for further study (**Table S1**).

Genomic DNA was extracted from selected isolates of *Diaporthe* spp. using E.Z.N.A. Fungal DNA mini kit (Omega Bio-tek, Darmstadt, Germany) from 100 mg of mycelium grown on PDA according to the manufacturer's instructions. Partial regions of three loci were amplified. The primer sets ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′)/ITS4 (5′-TCCGCTTATTGATATGC-3′) (White et al. 1990) were used to amplify the internal transcribed spacer (ITS) of ribosomal DNA. Primer pair EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′)/EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′) (Carbone and Kohn 1999) were used to amplify partial translation elongation factor 1-α gene (*tef1-α*), and the beta-tubulin (*tub2*) gene was amplified using primers Bt2a (5′ GGTAACCAAATCGGTGCTGCTTTC 3′) and Bt2b (5′ ACCCTCAGTGTAGTGACCCTTGGC 3′). In case of lack of amplification of beta-

tubulin gene, T1 (5′-AACATGCGTGAGATTGTAAGT-3′) and Bt2b (5′-ACCCTCA-GTGTAGTGACCCTTGGC-3′) primers were used (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997). The PCR amplification mixtures and cycling conditions for all three genes were performed according to Guarnaccia and Crous (2018). The amplification products were analyzed on 1% agarose (VWR Life Science AMRESCO® Biochemicals) after staining with GelRed™. PCR products were purified with the PCR Purification Kit (QIAquick®, Germany) before sequencing by Macrogen Europe B. V. (Amsterdam, Netherlands). The obtained sequences were analyzed and using the Geneious v. 11.1.5 program (Auckland, New Zealand).

Phylogenetic analysis

The sequences obtained from the 40 strains in this study were subjected to a blast search in NCBI's GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) nucleotide database to determine the closest species for a taxonomic framework of the studied isolates. The results of Blast analysis indicated that all the isolates belonged to *Diaporthe* genus. The sequences generated in this study and reference sequences of *Diaporthe* spp. (Hilário et al. 2021) were initially aligned by using the MAFFT v. 7 online servers (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley 2013), and then manually adjusted in MEGA v. 7 (Kumar et al. 2016). To achieve the sub-genus identification of the *Diaporthe* spp., phylogenetic analysis was performed first individually for each gene and then as a multilocus analysis of three loci (ITS, *tef-1α* and *tub2*). The phylogeny was based on Bayesian Inference (BI) and Maximum Parsimony (MP) for the multi-locus analysis (**Fig. 2**). For BI, the best evolutionary model for each locus was determined using MrModeltest v. 2.3 (Nylander 2004) and incorporated 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 at 0.2 and trees were sampled every 1,000 generations. The analysis stopped when the average standard deviation of split frequencies was below 0.01. The MP analysis was performed using Phylogenetic Analysis Using Parsimony (PAUP) 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', with all characters equally weighted 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 bootstrap analysis (Hillis and Bull 1993) were based on 1,000 replications. Sequences generated in this study were deposited in GenBank (**Table S2**).

Prevalence and distribution of *Diaporthe* spp.

The prevalence of *Diaporthe* spp. in hazelnut nuts collected from different sites in the investigated area was calculated according to Hilário et al. (2021). The Isolation Rate (RI) was calculated for each species using the formula:

$$RI\ \% = [NS / NI] \times 100$$

where NI was the total number of *Diaporthe* isolates collected during survey, NS was the number of isolates belonging to same species. Overall isolation rate was also determined by using the NI value that was equal total number of isolates from hazelnut nuts.

Pathogenicity trial

Pathogenicity of 40 *Diaporthe* strains was evaluated on detached ripening hazelnut nuts 'Tonda Gentile del Piemonte' (BBCH: 85; over 50% of the shells changed color) (Table S3). Nuts were surface disinfected with 1% NaClO and a piece of shell

(5 mm diameter) was removed with a sterile cork borer. A mycelium plug of 5 mm in diameter was taken from seven days old PDA colony and placed with the mycelium in contact with the nuts inside each well in the shell. Each inoculation point of nut was wrapped with Parafilm to avoid dehydration. Three replicates per isolate and three nuts per replicate were tested. Nine control nuts were treated with a sterilized PDA plugs as described above and served as negative control. Inoculated nuts were placed in plastic trays and covered with plastic foil and inoculated at 22±2 °C for 30 days in a chamber, with a 12 h light/12 h dark period each day. At the end of the incubation period disease severity was calculated by using a disease rating scale from 0 to 4 where 0= no visible symptoms, 1= <25% development of pycnidia, 2= 25-50% development of pycnidia, 3=50-75% development of pycnidia 4=≥75% development of pycnidia (**Fig. 3**).

Analysis of variance (ANOVA) was utilized for statistical analysis. The mean values were separated by Tukey HSD ($P < 0.05$) by using SPSS software (IBM SPSS Statistics v. 28.0.1.0).

Results

Fungal isolation and identification

On hazelnut nuts with defects, three types of symptoms were observed: black rot, moldy, and necrotic spots. Kernels were covered by white-grey mold and a few of them were totally rotten, wet, and almost black (**Fig. 1**). Fungi were identified based on their symptoms type, morphology (conidial morphology, colony shape, and color), and, when necessary, by using ITS sequencing. The prevalent fungi isolated from symptomatic hazelnut kernels were *Diaporthe* spp. (38%), *Botryosphaeria dothidea* (26%), and *Diplodia seriata* (14%). Moreover, *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., Mucorales, *Neofusicoccum* spp., *Penicillium* spp., and *Trichothecium roseum* were isolated from nuts but with incidence lower than 6%.

Diaporthe spp. were isolated from moldy nuts (68%), followed by necrotic nuts (17%) and black rotted nuts (15%). Members of Botryosphaeriaceae and *Alternaria* spp. were isolated only from black rotted and necrotic nuts. Whilst *Fusarium* spp. and *Trichothecium roseum* were isolated only from moldy nuts. Other fungal pathogens were isolated from mixed nuts (moldy, necrotic or black rotted).

Diaporthe spp. were isolated from all locations with different frequency: Lu and Cuccaro (30%), Murazzano (82%), Marsaglia (64%), Rodello (47%), Belvedere Langhe (32%), Cravanzana (36%), Cortemilia (50%), Borgo D'Ale (8%), and Cavaglià (42%) (**Table 1**).

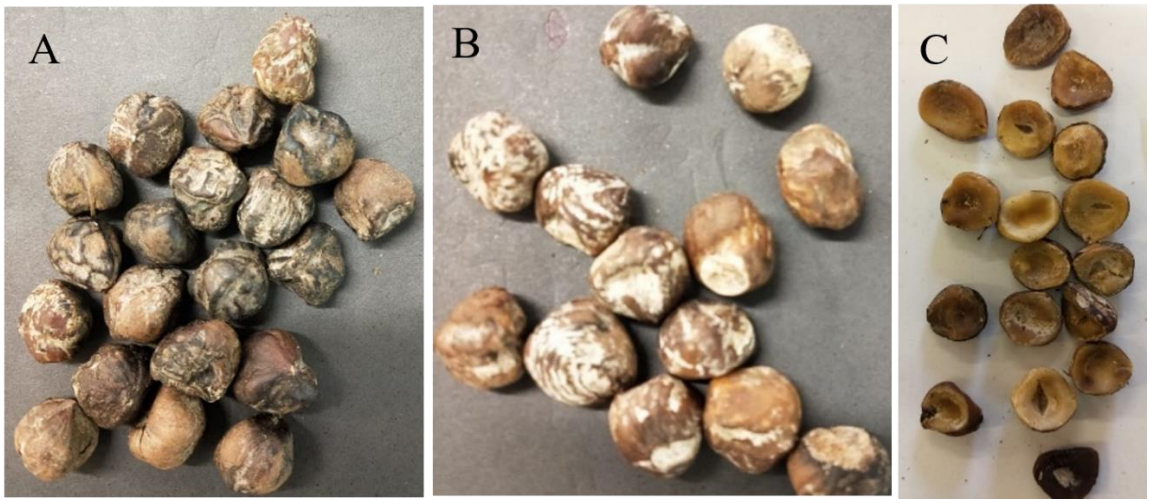


Fig. 1. Symptoms observed on hazelnut (*Corylus avellana*) described as (A) black rot (B) moldy (C) necrotic

Table 1: Overall incidence of all *Diaporthe* spp. on hazelnut nuts (*Corylus avellana*) variety and date of cultivation from different towns in Piedmont, Italy

Sr. No.	Towns/Province [1]	Variety [2]	Date of orchard planting [3]	*Diaporthe* incidence (%)
1	Lu and Cuccaro (AL)	TGP	NA	30.43
2	Murazzano (CN)	TGP	2010	82
3	Marsaglia (CN)	TGP	1995	63.6
4	Rodello (CN)	TGP	1990	47
5	Cravanzana (CN)	TGP	1995	36.3
6	Cortemilia (CN)	TGP	1980	50
7	Borgo D'Ale (VC)	TGP	2011	8.3
8	Cavaglià (BI)	TGP	2013	42.4
9	Belvedere Langhe (CN)	TGP	1975	32

[1] Provinces are administrative areas in Italy: AL (Alessandria); CN (Cuneo); BI (Biella); VC (Vercelli).

[2] TGP: 'Tonda Gentile del Piemonte' [3]

NA: not available

Phylogenetic analysis

To further explore the diversity of the *Diaporthe* spp. isolates and to assign the species, three genes were sequenced: ITS, *tub2*, *tef-1α*, A combined alignment of the three loci was analyzed. The combined alignment consisted of 111 sequences including the outgroup *Diaporthella corylina* (CBS 121124). The final dataset comprised of 1,362 nucleotides (ITS: 1-473, *tub2*: 480-876, *tef-1α*: 883-1,362). A total of 559 nucleotides were parsimony-informative, 194 were variable and parsimony-uninformative, and 597 were constant. A maximum of 1,000 equally MP trees were saved (Tree length = 3049, CI = 0.471, RI = 0.872, and RC = 0.410). Bootstrap support values from the MP analysis are included on the Bayesian tree in **Fig. 2**. For the BI, MrModeltest suggested that all partitions should be analyzed with Dirichlet state frequency distributions. The following models were recommended by MrModeltest and used: GTR+I+G for ITS and *tef-1α*, and HKY+G for *tub2*. In the BI, the ITS partition had 179 unique site patterns, the *tef-1α* partition had 350 unique site patterns, the *tub2* partition had 259 unique site patterns and the analysis ran for 3,495,000 generations, resulting in 3,496 trees of which 2,622 trees were samples to calculate the posterior probabilities. Multi-locus phylogenetic analysis of 40 isolates showed that 28 isolates clustered with *D. eres*, 3 isolates clustered with *D. novem*, 3 with *D. foeniculina*, and 3 with *D. rudis*. Hm-20a-2, HBr-9b-1 and HM-30-2 formed distinct lineage with *D. oncostoma*, *D. ravennica*, and *D. sojae*, respectively.

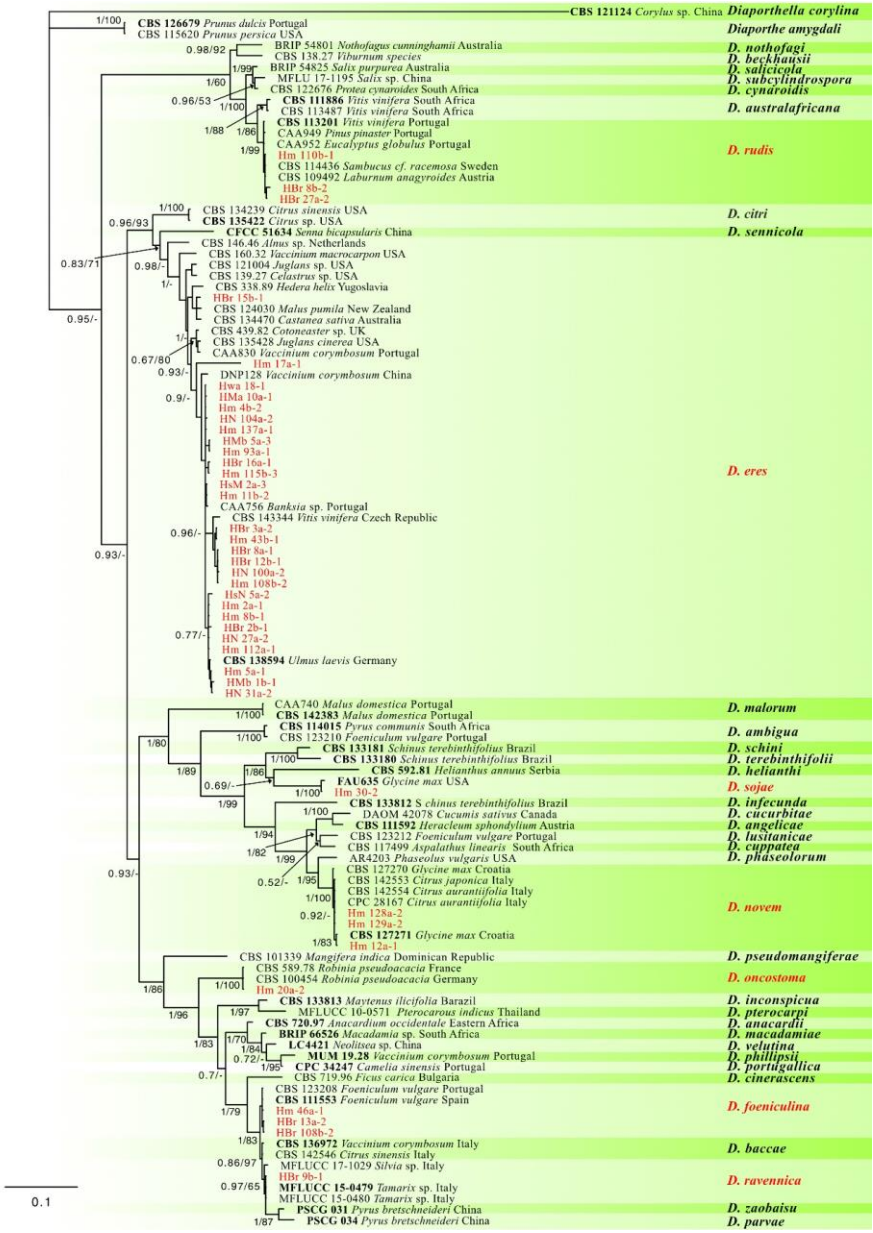


Fig. 2. Consensus phylogram of 2,622 trees resulting from a Bayesian analysis of the combined ITS, *tef-1α*, and *tub2* sequence alignments of the *Diaporthe* species. Bootstrap support values and Bayesian posterior probability values are indicated at the nodes. Host and country of origin are listed next to the strain numbers. Ex-type isolates are indicated in bold. The isolates obtained in this study are in red. The tree was rooted with *Diaporthella corylina* (CBS 121124).

Morphology

Morphological observations from edges of the Petri dishes, supported by phylogenetic inference, were used to describe the seven species of *Diaporthe* spp. (**Fig. 4 A-I**).

Colonies of *Diaporthe sojae* growing on PDA reached 90 mm within 10 days at 25-26 °C. Colonies were white, with fluffy aerial mycelium. Reverse colonies were creamy and later developed a yellowish pigmentation in center of the Petri dish. Alpha-conidia were hyaline, aseptate, abundant, smooth, ellipsoidal and biguttulate with dimensions 5.8-7.3x2-3.2 µm mean ± SD = 6.67±0.78x2.57±0.60 µm (**Fig. S1 A-B; Fig. S2 A**).

Ten days colonies of *Diaporthe foeniculina* growing on PDA reached 90 mm at 25-26 °C. Colonies were white with fluffy aerial mycelium. Reverse colonies had green to yellowish pigmentation. Alpha conidia were hyaline, aseptate, smooth, ellipsoidal or fusiform, with one or many guttules with dimensions 7.6-9.5x2.5-2.9 µm mean ± SD = 8.6±0.96x2.7±0.21 µm (**Fig. S1 C.D; Fig. S2 B**).

Diaporthe novem had ten-day colonies on PDA reaching 90 mm at 25-26 °C. Colonies were flat, fluffy, and white to greyish colonies in center with white aerial mycelium at the margins of the Petri dish. Colony reverse had black circles and white margins. Alpha-conidia were hyaline, unicellular, oval to cylindrical, biguttulate with obtuse ends, with dimensions of 6.9-9.1x2.5-2.9 µm, mean ± SD =8.1±1.11x2.73±0.21 µm (**Fig. S1 E-F; Fig. S2 C**).

Ten days colonies of *Diaporthe ravennica* growing on PDA reached 90 mm at 25-26 °C. Colonies were white, spreading to the edge in wavy appearance, with pycnidia production in middle of plate, medium flat or dense. Reverse colony was creamy, radiating white outwardly with black dots in middle. Alpha-conidia were smooth, hyaline, aseptate, multi-guttulate, ovate to ellipsoidal with dimensions 8.3-11.9x2.5-4.3 µm mean ± SD =10.1±1.80x3.30±0.92 µm (**Fig.S1 G-H; Fig. S2 D**).

Colonies of *Diaporthe rudis* growing on PDA reached 85 mm within 4 weeks at 25-26 °C. Colonies were white and turn brown gradually, fluffy, and flat, radiating outwardly to the edge, with brown mycelium. Colony reverse was greyish with brown halos. Alpha conidia were ellipsoidal, hyaline, biguttulate and smooth. Dimensions of conidia were 7.5-8.5x2.4-2.7 μm mean ± SD =7.97±0.50x2.57±0.15 μm (**Fig. S1 I-J; Fig. S2 E**).

Colonies of *Diaporthe eres* were white greyish, fluffy aerial mycelium with abundant pycnidia at maturity. Colony reverse had dark pigmentation in center and whitish from edges of the Petri dish. Colonies growing on PDA reached 90 mm at 25-26 °C after 10 days. Alpha conidia were ovate to ellipsoidal, aseptate, smooth, hyaline, biguttulate, and base subtruncate with dimensions of 8.5-11.1x 3.5-4.5 μm, mean ± SD =9.7±1.31x3.67±0.76 μm (**Fig. S1 K-L; Fig. S2 F**).

Colonies of *Diaporthe oncostoma* were smooth and flat with no exudate. Dense fluffy mycelium was observed. Colony reverse had yellowish green and periphery umber. Colonies growing on PDA reached 25 mm at 25-26 °C after 10 days. Alpha conidia were hyaline, septate, and bent cylindrical with dimensions of 11-14.5x 3.2-4.9 μm, mean ± SD = 12.83±1.76x4.20±0.89 μm (**Fig. S1 M-N; Fig. S2 C**).

Prevalence and distribution of *Diaporthe* spp.

Overall, *Diaporthe* spp. were isolated from all the sites surveyed (Lu and Cuccaro, Murazzano, Marsaglia, Rodello, Belvedere Langhe, Cravanzana, Cortemilia, Borgo D'Ale, and Cavaglià) showing symptoms of black rot, moldy, and necrosis. In particular, *Diaporthe* spp. were predominantly isolated from moldy nuts. Based on morphology, among 161 *Diaporthe* spp. isolates, *D. eres* was the dominant species, representing 92.5 % of the isolates collected. Three strains per species of *D. rudis*, *D. novem*, and *D. foeniculina* were isolated. Only one isolate was found per each of the species *Diaporthe oncostoma*, *D. sojae*, and *D. ravennica*.

Diaporthe eres was predominantly isolated in all the geographical areas: it was the only species isolated in Belvedere Langhe, Cravanzana, Cortemilia, and Borgo D'Ale. Moreover, it represented the most isolated species in the other geographical areas: 94 %, 88 %, 93 %, 88 %, and 86% of the collected isolates were *D. eres* in Lu and Cuccaro, Murazzano, Marsaglia, Rodello, and Cavaglià, respectively. Whilst *D. rudis* and *D. novem* were recovered only from three and two towns, respectively, with an abundance lower than 15 % (**Fig. 5**).

Pathogenicity test

After fifteen days, all the *Diaporthe* isolates developed lesions on the surface of inoculated kernels. Moreover, the inoculated kernels showed abundant development of pycnidia with different disease severity (disease rating scale 0-4) on the whole nut as shown in **Fig. 3 and Table S3**. In a few cases internal black discoloration was also observed on nuts along with necrotic spots of variable sizes. All the *Diaporthe* isolates were successfully reisolated from all the inoculated nuts, fulfilling Koch's postulates. Control hazelnut kernels showed no internal or external symptoms.

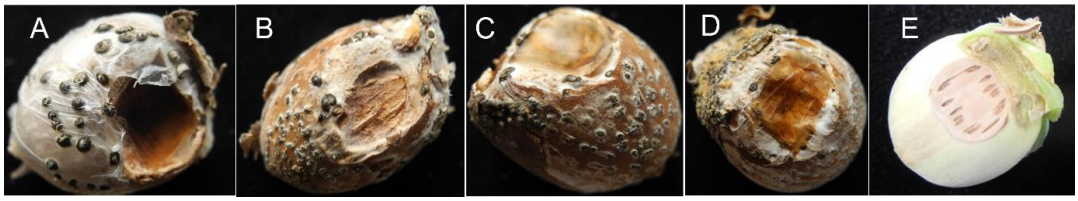


Fig. 3. Symptomatology induced on hazelnut nuts 'Tonda Gentile del Piemonte' inoculated with *Diaporthe* spp. and stored at 22±2 °C for 30 days in a chamber with a 12 h light/12 h dark. Symptoms of nut rot were evaluated by the following scale: 0: no visible symptoms **(E)**; 1: <25% development of pycnidia **(D)**; 2: 25-50% development of pycnidia **(C)**; 3: 50-75% development of pycnidia **(B)**; 4: ≥75% development of pycnidia **(A)**.

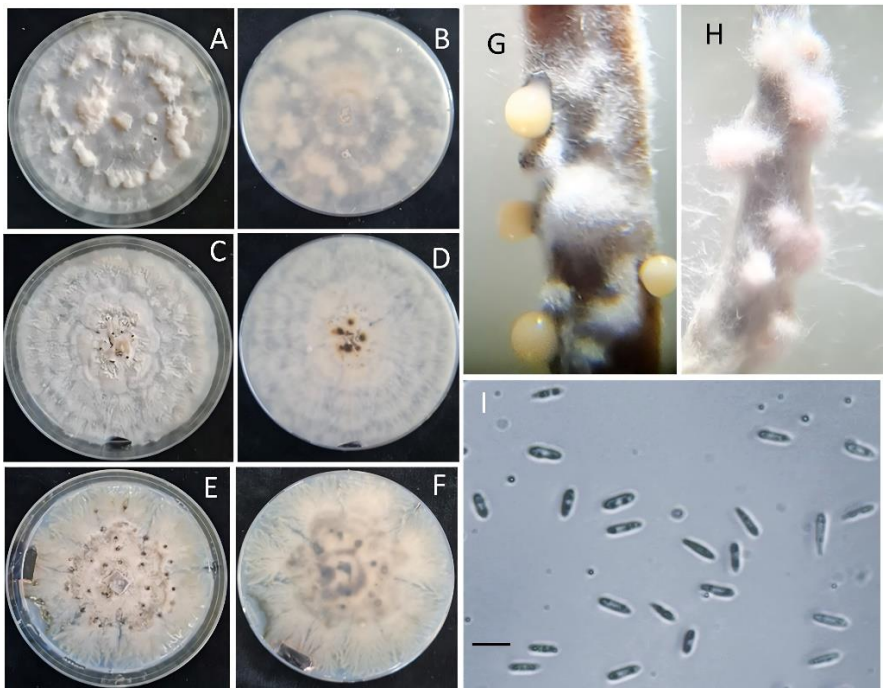


Fig. 4 (A-B) Morphological features of *Diaporthe* spp. obtained from hazelnut nuts **(A-B)** *D. sojae*. Front and reverse side of colony grown on PDA after 10 days at 23 ± 2 °C**(C-D)** *D. ravennica*. Front and reverse side of colony grown on PDA **(E-F)** *D. eres*. Front and reverse side of colony grown on PDA after 10 days at 23 ± 2 °C. **(G)** Conidiomata of *D. eres* on sterilized pine needle on WA. **(H)** Conidiomata of *D. sojae* on sterilized pine needle on WA **(I)** Conidia of *D. eres* (dimensions: 8.5-11.1x 3.5-4.5 µm; scale bar: 10 µm).

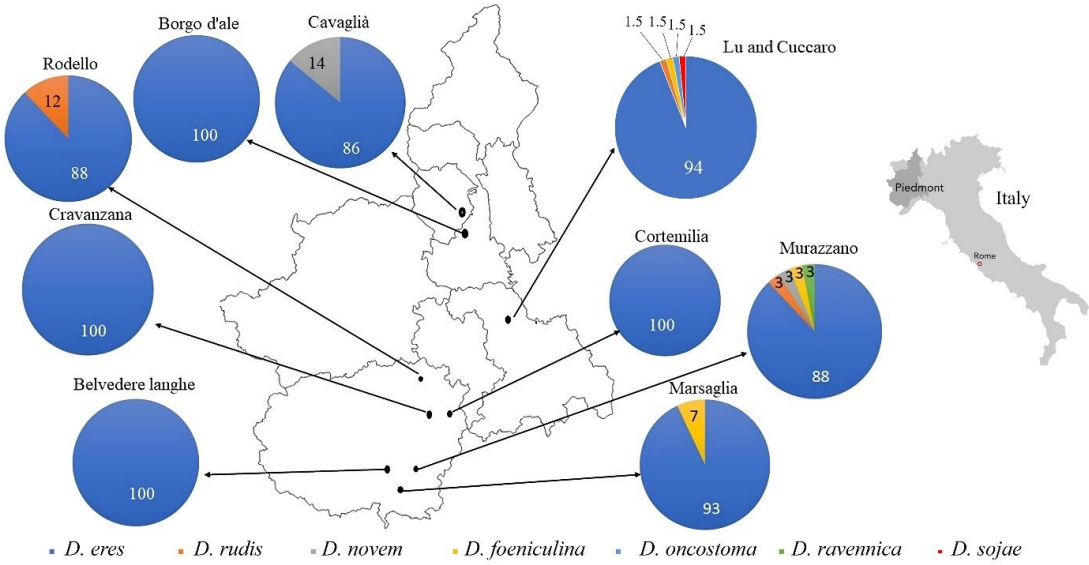


Fig. 5. Incidence (%) and distribution of *Diaporthe* species from hazelnut nuts (*Corylus avellana*) according to their sampling locations of Piedmont, Italy.

Discussion

Hazelnut kernel defects are a serious threat for hazelnut yield, quality, and market value. The present study aimed to investigate the causal agents and the diversity of fungal species associated with rotten hazelnut nuts in Italy. During the survey performed from 2020 to 2021 in northern Italy, we isolated and identified *Alternaria* spp., *Aspergillus*, *B. dothidea*, *Diaporthe* spp., *D. seriata*, *Fusarium* spp., Mucorales, *Neofusicoccum* spp., *Penicillium*, and *T. roseum* based on morphological and ITS analysis. Distinct taxonomic groups were isolated from different categories of symptoms. Members of Botryosphaeriaceae and *Alternaria* spp. were isolated only from black rotted and necrotic nuts. Whilst *Fusarium* spp. and *Trichothecium roseum* were isolated only from moldy nuts. Previously, *N. parvum* (Waqas et al. 2022), *Alternaria* spp. (Battilani et al. 2018; Belisario et al. 2004), *B. dothidea* and *D. seriata* (Luna et al. 2022) were isolated from rotten hazelnuts or other nut crops. *Diaporthe* spp. were the most dominant fungi isolated from defected hazelnut nuts in northern Italy. Most *Diaporthe* spp. were isolated from moldy nuts (68%), but also from necrotic and black rotted nuts. The prevalence of *Diaporthe* spp. associated with nut rot is closely correlated to the sampling area and to the type of nut symptoms. The highest diversity of *Diaporthe* spp. was observed in Murazzano, where 51% nuts were moldy which is in agreement with previous studies on hazelnuts from Georgia and Turkey (Battilani et al. 2018; Arciuolo et al. 2020) *Diaporthe* spp. have a wide host range (Gomes et al. 2013; Lombard et al. 2014; Udayanga et al. 2015; Yang et al. 2021) and were previously reported as causal agents of diseases of hazelnut nuts (Arciuolo et al. 2020; Bai et al. 2022; Battilani et al. 2018; Gao et al. 2021; Guerrero et al. 2020) and other nut crops (Eichmeier et al. 2020; Lawrence et al. 2015; León et al. 2020; Yang et al. 2018). Previously, *Diaporthe* spp. were isolated from hazelnut nuts and identified at genus level in Italy (Vitale et al. 2020).

ITS sequence is commonly used for species identification of Diaporthaceae and Botryosphaeriaceae (Hyde et al. 2014; Marin-Felix et al. 2019). However, ITS sequence is not informative to distinguish the *Diaporthe* species because of greater intraspecific variation in ITS locus as compared to interspecific variation (Chaisiri et al. 2021; Santos et al. 2010). Therefore, a multi-locus phylogenetic analyses approach is used for accurate identification and resolution of *Diaporthe* species (Guarnaccia et al. 2020; Lesuthu et al. 2019; Santos et al. 2017; Zapata et al. 2020). DNA sequence data combined with morphology has been extensively used to establish the species boundaries in *Diaporthe* genus (Gao et al. 2017; Hilario et al. 2021).

Seven *Diaporthe* spp. were identified in this study based on morphological features and three genomic regions ITS, *tef-1α* and *tub2*. The closest taxa of seven *Diaporthe* spp. recovered from this study have been included in the analysis, based on BLASTn search in NCBI's GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) nucleotide database. The final multilocus phylogenetic tree differentiate the *D. eres*, *D. foeniculina*, *D. novem*, *D. oncostoma*, *D. ravennica*, *D. rudis*, and *D. sojae* on hazelnut nuts.

D. eres was the most frequently isolated species associated with defected hazelnut nuts from all the areas of northern Italy. *D. eres* is an important plant pathogen which can infect various hosts (Bai et al. 2022) and has been considered as the main causal agent of hazelnut nuts defects in the Caucasus region (Arciuolo et al. 2020; Battilani et al. 2018). Although *D. eres* was reported on hazelnut nuts in Italy and Turkey by Arciuolo et al. (2020), most isolates could not be classified at species level due to the poorly supported or non-monophyletic clade. Moreover, pathogenicity of *D. eres* isolates was unknown on hazelnut nuts. However, our analysis based on three loci, combined with morphological observations, clearly identified *D. eres* at species level. Previously, *D. eres* was associated with hazelnut canker in China and the USA (Bai et al., 2022; Gao et al., 2021; Wiman et al. 2019), and on other hosts

(Guarnaccia et al. 2018; Hilário et al. 2021; Lombard et al. 2014; Udayanga et al. 2014; Wang et al. 2021).

Previous studies showed that *D. foeniculina* and *D. rudis* can be pathogenic or saprophytic on different host plants (Gajanayake et al. 2020; Marin-Felix et al. 2019; Udayanga et al. 2014). Recently, Guerrero et al. (2020) described *D. foeniculina* associated with black tip and necrotic spots on hazelnut kernel in Chile and stem and shoot cankers on sweet chestnuts in Italy (Annesi et al. 2016). Moreover, kernel mold of hazelnut was caused also by *D. rudis* in the USA (Pscheidt et al. 2019) and on other hosts in Italy (Dissanayake et al. 2017; Guarnaccia et al. 2020). Similarly, we detected these species associated with nut rot of hazelnut nuts in Italy. Moreover, *D. sojae* was isolated and identified for the first time from infected nuts of hazelnut in Italy, where it was already reported on hazelnut in Turkey (Arciuolo et al. 2020) and on *Glycine soja* in Italy (Gomes et al. 2013). There are no previous records of *D. novem*, *D. ravennica,* and *D. oncostoma* on hazelnut nuts. So, this study suggests that hazelnut could be a host for *D. novem*, *D. ravennica,* and *D. oncostoma.* Previously, *D. novem* has been reported on *Citrus* spp. in Italy (Guarnaccia and Crous 2017) and on almond in the USA (Lawrence et al. 2015), whilst *D. ravennica* and *D. oncostoma* have been reported on *Salvia* sp. and *Tamarix* sp. in Italy (Dissanayake et al. 2017; Thambugala et al. 2017) and *Robinia pseudoacacia* by Gomes et al. (2013) respectively.

In our phylogeny, the three loci used in this study were unable, when considered singularly, to discriminate *D. revennica* from *D. foeniculina*, and *D. baccae*. Separation of *D. ravennica* from *D. foeniculina*, and *D. baccae* was possible only with a combined analysis of ITS, *tef-1α*, and *tub2* regions as reported in previous studies (Aiello et al. 2022; Gajanayake et al. 2020; Phukhamsakda et al. 2020). *D. sojae* strain also formed a well-supported clade with CBS 139282 (Udayanga et al. 2015). Our results of phylogeny showed a large diversity of *Diaporthe* spp.

comprising several clades and species, associated with nut rot of hazelnut nuts in Italy.

The pathogenicity tests showed that all the studied isolates were pathogenic on hazelnut nuts, resulting in different disease severity. A total of 40 strains of *Diaporthe*, belonging to *D. eres*, *D. foeniculina*, *D. novem*, *D. oncostoma*, *D. ravennica*, *D. rudis*, and *D. sojae* were able to cause nut rot of hazelnut. *Diaporthe eres* was the most virulent species as compared to the other species of *Diaporthe* which is in agreement with previous studies (Arciuolo et al. 2020; Bai et al., 2022; Battilani et al. 2018; Gao et al., 2021). Whereas *D. oncostoma* was comparatively less pathogenic (smaller lesions), suggesting that *D. oncostoma* is probably a weak pathogen on hazelnut nuts. The other species were moderately pathogenic on their original host.

In conclusion, this study elucidates the species of *Diaporthe* associated with defected hazelnut nuts in northern Italy, by using morphology analysis, molecular data, and provides information about the pathogenicity. This is the first report worldwide about nut rot of hazelnut caused by *D. novem, D. oncostoma*, and *D. ravennica*, and the first report of *D. foeniculina*, *D. rudis*, and *D. sojae* as agents of nut rot of hazelnut in Italy.

In this study, we also observed the co-occurrence of *Diaporthe* spp. in the same nuts with members of Botryosphaeriaceae, as previously reported (Elfar et al. 2013; Guarnaccia et al. 2016; Moral et al. 2017). Such co-existence is not new, as it has been previously described on branch cankers and stem-end rot of different hosts in Italy and on hazelnut nuts in Caucasus region (Battilani et al. 2018; Guarnaccia et al. 2016; 2020). Furthermore, *Diaporthe* spp. are cosmopolitan fungi and can infect a range of cultivated crops and natural ecosystem as a plant pathogens or saprophytes. Taking all of this into consideration, it deserves further investigation that *Diaporthe* spp. with other members of Botryosphaeriaceae, may contribute to

cause fungal trunk disease (FTD) of hazelnuts in Italy, that could be a threat to European hazelnut production.

Future studies should also focus on elucidating the epidemiology of this disease and on the climatic conditions that favor the development of *Diaporthe* spp. on hazelnut. Prevention and control measures should target *D. eres*, which showed to be the main responsible species of defects on hazelnuts in Italy.

e-Xtra (Supplementary) Files

Table S1.

Isolates of *Diaporthe* spp. across nine regions of Piedmont, Italy, obtained from hazelnut (*Corylus avellana*) during 2020-2021 and their GenBank accession numbers.

Sr. no.	Isolate code	Fungal species	Isolation year	Towns/Provinces[1]	Altitude	GenBank Accession [2]		
						ITS	*tef1-α*	*tub2*
1	Hwa-18-1	*Diaporthe eres*	2020	Lu and Cuccaro (AL)	307 m	OM331706	ON933990	OP186258
2	HsN5a-2	*D. eres*	2020	Lu and Cuccaro (AL)	307 m	OM331707	ON933991	OP186259
3	HsM2a-3	*D. eres*	2020	Lu and Cuccaro (AL)	307 m	OM331708	ON933992	OP186260
4	HMb-1b-1	*D. eres*	2020	Lu and Cuccaro (AL)	307 m	OM331709	ON933993	-
5	HMb-5a-3	*D. eres*	2020	Lu and Cuccaro (AL)	307 m	OM331710	ON933994	OP186261
6	Hma-10a-1	*D. eres*	2020	Lu and Cuccaro (AL)	307 m	OM331711	ON933995	OP186262
7	HM-2a-1	*D. eres*	2020	Murazzano (CN)	739 m	OM331712	ON933996	OP186263
8	HM-4b-2	*D. eres*	2020	Murazzano (CN)	739 m	OM331713	ON933997	OP186264
9	HM-5a-1	*D. eres*	2020	Murazzano (CN)	739 m	OM331714	ON933998	OP186265
10	HM-8b-1	*D. eres*	2020	Murazzano (CN)	739 m	OM331715	ON933999	OP186266
11	HM-11b-2	*D. eres*	2020	Murazzano (CN)	739 m	OM331716	ON934000	OP186267
12	Hm-12a-1	*D. novem*	2020	Murazzano (CN)	739 m	OM331738	ON933987	OP156876
13	HM-17a-1	*D. eres*	2020	Murazzano (CN)	739 m	OM331717	ON934001	OP186268

14	Hm-20a-2	*D. oncostoma*	2020	Murazzano (CN)	739 m	ON911324	ON934002	OP186269
15	HBR-2b-1	*D. eres*	2020	Murazzano (CN)	739 m	OM331718	OP288105	OP186270
16	HBr-3a-2	*D. eres*	2020	Murazzano (CN)	739 m	ON911327	OP296410	OP186271
17	HBR-8a-1	*D. eres*	2020	Murazzano (CN)	739 m	OM331719	ON934003	OP186272
18	HBr-8b-2	*D. rudis*	2020	Murazzano (CN)	739 m	OM331733	ON934004	OP186273
19	HBr-9b-1	*D. ravennica*	2020	Murazzano (CN)	739 m	ON911325	ON934005	OP186274
20	HBR-12b-1	*D. eres*	2020	Murazzano (CN)	739 m	OM331720	ON934006	OP186275
21	HBr-13a-2	*D. foeniculina*	2020	Murazzano (CN)	739 m	ON911322	ON934007	OP186276
22	HBR-15b-1	*D. eres*	2020	Murazzano (CN)	739 m	OM331721	ON934008	OP186277
23	HBR-16a-1	*D. eres*	2020	Murazzano (CN)	739 m	OM331722	ON934009	OP186278
24	HN-27a-2	*D. eres*	2021	Lu and Cuccaro (AL)	307 m	OM331723	ON934010	OP186279
25	HBr-27a-2	*D. rudis*	2021	Lu and Cuccaro (AL)	307 m	OM331734	ON934011	OP186280
26	Hm-30-2	*D. sojae*	2021	Lu and Cuccaro (AL)	307 m	ON911326	ON934012	OP186281
27	HN-31a-2	*D. eres*	2021	Lu and Cuccaro (AL)	307 m	OM331724	ON934013	OP186282
28	HM-43b-1	*D. eres*	2021	Lu and Cuccaro (AL)	307 m	OM331725	ON934014	OP186283
29	Hm-46a-1	*D. foeniculina*	2021	Lu and Cuccaro (AL)	307 m	ON911323	ON934015	-
30	HM-93a-1	*D. eres*	2021	Cortemilia (CN)	247 m	OM331726	ON934016	OP186284
31	HN-100a-2	*D. eres*	2021	Cravanzana (CN)	580 m	OM331727	ON934017	OP186285
32	HM-108b-2	*D. eres*	2021	Marsaglia (CN)	550 m	OM331728	ON934018	OP186286

33	HN-104a-2	*D. eres*	2021	Belvedere Langhe (CN)	679 m	OM331729	ON934019	OP186287
34	Hm-110b-1	*D. rudis*	2021	Rodello (CN)	600 m	OM331735	ON934020	OP186288
35	HM-112a-1	*D. eres*	2021	Rodello (CN)	600 m	OM331730	ON934021	OP186289
36	HM-115b-3	*D. eres*	2021	Rodello (CN)	600 m	OM331731	ON934022	OP186290
37	Hm-128a-2	*D. novem*	2021	Cavaglià (BI)	270 m	OM331737	ON933988	OP156877
38	Hm-129a-2	*D. novem*	2021	Cavaglià (BI)	270 m	OM331736	ON933989	OP156878
39	HBr-108b-2	*D. foeniculina*	2021	Cavaglià (BI)	270 m	ON911321	ON934023	OP186291
40	HM-137a-1	*D. eres*	2021	Borgo D'Ale (VC)	240 m	OM331732	-	OP186292

[1] AL: Alessandria, CN: Cuneo, BI: Biella, VC: Vercelli; [2] ITS: internal transcribed spacers 1 and 4 together with 5.8S nrDNA; *tef1-α*: translation elongation factor 1-α gene; *tub2*: beta-tubulin gene. Sequences generated in this study indicated in italics.

Table S2.
List of reference sequences of *Diaporthe* spp. including the outgroup *Diaporthella corylina* used in phylogenetic analyses for this study. Information includes GenBank accession numbers, strains, host, and origins.

Species	Strain [1]	Host	Country	GenBank Accession [2]		
				ITS	*tef1-α*	*tub2*
Diaporthe ambigua	CBS 114015^{T}	*Pyrus communis*	South Africa	KC343010	KC343736	KC343978
D. ambigua	CBS 123210	*Foeniculum vulgare*	Portugal	KC343012	KC343738	KC343980
D. amygdali	CBS 126679^{T}	*Prunus dulcis*	Portugal	KC343022	KC343748	KC343990
D. amygdali	CBS 115620	*Prunus persica*	USA	KC343020	KC343746	KC343988
D. anacardii	CBS 720.97^{T}	*Anacardium occidentale*	Eastern Africa	KC343024	KC343750	KC343992
D. angelicae	CBS 111592^{T}	*Heracleum sphondylium*	Austria	KC343027	KC343753	KC343995
D. australafricana	CBS 111886	*Vitis vinifera*	South Africa	KC343038	KC343764	KC344006
D. australafricana	CBS 113487^{T}	*Vitis vinifera*	South Africa	KC343039	KC343765	KC344007
D. baccae	CBS 136972^{T}	*Vaccinium corymbosum*	Italy	KJ160565	KJ160597	MF418509
D. baccae	CBS 142546	*Citrus sinensis*	Italy	MF418358	MF418437	MF418517
D. beckhausii	CBS 138.27	*Viburnum species*	Unknown	KC343041	KC343767	KC344009
D. citri	CBS 134239	*Citrus cinensis*	USA	KC357553	KC357522	KC357456
D. citri	CBS 135422^{T}	*Citrus* sp.	USA	KC843311	KC843071	KC843187
D. cinerascens	CBS 719.96	*Ficus carica*	Bulgaria	KC343050	KC343776	KC344018
D. cucurbitae	DAOM 42078	*Cucumis sativus*	Canada	KM453210	KM453211	KP118848
D. cuppatea	CBS 117499	*Aspalathus linearis*	South Africa	KC343057	KC343783	KC344025
D. cynaroidis	CBS 122676	*Protea cynaroides*	South Africa	KC343058	KC343784	KC344026

D. eres	CBS 138594^{T}	*Ulmus laevis*	Germany	KJ210529	KJ210550	KJ420799
D. eres	CBS 143344	*Vitis vinifera*	Czech Republic	MG281020	MG281541	MG281193
D. eres	CAA756	*Banksia* sp.	Portugal	MW040531	MW052385	MW091320
D. eres	CBS 146.46	*Alnus* sp.	The Netherlands	KC343008	KC343734	KC343976
D. eres	CBS 121004	*Juglans* sp.	USA	KC343134	KC343860	KC344102
D. eres	CBS 124030	*Malus pumila*	New Zealand	KC343149	KC343875	KC344117
D. eres	CBS 134470	*Castanea sativa*	Australia	KC343146	KC343872	KC344114
D. eres	DNP128	*Vaccinium corymbosum*	China	KC763096	KJ210561	KJ420801
D. eres	CBS 139.27	*Celastrus* sp.	USA	KC343047	KC343773	KC344015
D. eres	CBS 135428	*Juglans cinerea*	USA	KC843328	KC843121	KC843229
D. eres	CBS 439.82	*Cotoneaster* sp.	UK	KC343090	KC343816	KC344058
D. eres	CBS 338.89	*Hedera helix*	Yugoslavia	KC343152	KC343878	KC344120
D. eres	CBS 160.32	*Vaccinium macrocarpon*	USA	AF317578	GQ250326	KC344196
D. eres	MUM 19.31=CAA830	*Vaccinium corymbosum*	Portugal	MK792309	MK828080	MK837931
D. foeniculina	CBS 123208	*Foeniculum vulgare*	Portugal	KC343104	KC343830	KC344072
D. foeniculina	CBS 111553^{T}	*Foeniculum vulgare*	Spain	KC343101	KC343827	KC344069
D. helianthi	CBS 592.81^{T}	*Helianthus annuus*	Serbia	KC343115	KC343841	KC344083
D. inconspicua	CBS 133813^{T}	*Maytenus ilicifolia*	Brazil	KC343123	KC343849	KC344091
D. infecunda	CBS 133812^{T}	*Schinus terebinthifolius*	Brazil	KC343126	KC343852	KC344094
D. lusitanicae	CBS 123212	*Foeniculum vulgare*	Portugal	KC343136	KC343862	KC344104
D. macadamiae	BRIP 66526^{T}	*Macadamia* sp.	South Africa	MN708230	MN696528	MN696539

D. malorum	CAA740	*Malus domestica*	Portugal	KY435642	KY435629	KY435670
D. malorum	CBS 142383^{T}	*Malus domestica*	Portugal	KY435638	KY435627	KY435668
D. nothofagi	BRIP 54801	*Nothofagus cunninghamii*	Australia	JX862530	JX862536	KF170922
D. novem	CBS 127270	*Glycine max*	Croatia	KC343156	KC343882	KC344124
D. novem	CBS 127271^{T}	*Glycine max*	Croatia	KC343157	KC343883	KC344125
D. novem	CPC 26188 =CBS 142553	*Citrus japonica*	Italy	MF418426	MF418505	MF418586
D. novem	CPC 28165 = CBS 142554	*glycine max*	Italy	MF418427	MF418506	MF418587
D. novem	CPC 28167	*Citrus aurantiifolia*	Italy	MF418428	MF418507	MF418588
D. oncostoma	CBS 589.78	*Robinia pseudoacacia*	France	KC343162	KC343888	KC344130
D. oncostoma	CBS 100454	*Robinia pseudoacacia*	Germany	KC343160	KC343886	KC344128
D. parvae	PSCG 034^{T}	*Pyrus bretschneideri*	China	MK626919	MK654858	MK691248
D. phaseolorum	CBS 139281=AR4203	*Phaseolus vulgaris*	USA	KJ590738	KJ590739	KJ610893
D. phillipsii	MUM 19.28^{T}	*Vaccinium corymbosum*	Portugal	MK792305	MK828076	MN000351
D. portugallica	CBS 144228=CPC34247^{T}	*Camelia sinensis*	Portugal	MH063905	MH063911	MH063917
D. pseudomangiferae	CBS 101339= MFLU 15-3228^{T}	*Mangifera indica*	Dominican Republic	KC343181	KC343907	KC344149
D. pterocarpi	MFLUCC 10-0571	*Pterocarous indicus*	Thailand	JQ619899	JX275416	JX275460
D. ravennica	MFLUCC 17-1029	*Salvia* sp.	Italy	KY964191	KY964147	KY964075
D. ravennica	MFLUCC 15-0479^{T}	*Tamarix* sp.	Italy	KU900335	KX365197	KX432254
D. ravennica	MFLUCC 15-0480	*Tamarix* sp.	Italy	KU900336	KX426703	KX377688

D. rudis	CBS 114436	*Sambucus cf. racemosa*	Sweden	KC343236	KC343962	KC344204
D. rudis	CBS 109492	*Laburnum anagyroides*	Austria	KC343232	KC343958	KC344200
D. rudis	CBS 113201^{T}	*Vitis vinifera*	Portugal	KC343234	KC343960	KC344202
D. rudis	CAA949	*Pinus pinaster*	Portugal	MN190304	MT309426	MT309452
D. rudis	CAA952	*Eucalyptus globulus*	Portugal	MN190307	MT309429	MT309455
D. salicicola	BRIP 54825	*Salix purpurea*	Australia	JX862531	JX862537	KF170923
D. schini	CBS 133181T	*Schinus terebinthifolius*	Brazil	KC343191	KC343917	KC344159
D. sennicola	CFCC 51634	*Senna bicapsularis*	China	KY203722	KY228883	KY228889
D. sojae	CBS 139282=FAU635^{T}	*Glycine max*	USA	KJ590719	KJ590762	KJ610875
D. subcylindrospora	MFLU 17-1195	*Salix* sp.	China	MG746629	MG746630	MG746631
D. terebinthifolii	CBS 133180^{T}	*Schinus terebinthifolius*	Brazil	KC343216	KC343942	KC344184
D. velutina	CGMCC 3.18286=LC4421^{T}	*Neolitsea* sp.	China	KX986790	KX999182	KX999223
D. zaobaisu	PSCG031^{T}	*Pyrus bretschneideri*	China	MK626922	MK654855	MK691245
Diaporthella corylina	CBS 121124^{T}	*Corylus* sp.	China	KC343004	KC343730	KC343972

[1] BRIP: Plant Pathology Herbarium, Department of Primary Industries, Dutton Park, Queensland, Australia CAA: Culture Collection Artur Alves, University of Aveiro, Aveiro, Portugal; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CFCC: China Forestry Culture Collection Center, Beijing, China; CGMCC: China, General Microbiological Culture Collection, Beijing, China; CMW: Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CPC: Culture collection of P.W. Crous, housed at Westerdijk Fungal Biodiversity Institute; DAOMC: The Canadian Collection of Fungal Cultures, Canada; DNP: Isolates in SMML culture collection, USDA-ARS, Beltsville, USA; FAU:

Isolates in culture collection of Systematic Mycology and Microbiology Laboratory, USDA-ARS, Beltsville, MD, USA; LC: Working collection of Lei Cai,housed at Institute of Microbiology, Chinese; MFLU: Herbarium of Mae Fah Luang University, Thailand; MIFCC: Michigan Isolate Fungal Culture Collection, Michigan, USA; MUM: Culture Collection from Micoteca da Universidade do Minho, Center for Biological Engineering of University of Minho, Braga, Portugal. Ex-type isolates are indicated with ^{T}.

[2] ITS: internal transcribed spacers 1 and 4 together with 5.8S nrDNA; *tef1-α*: translation elongation factor 1-α gene; *tub2*: beta-tubulin gene.

Sequences generated in this study indicated in italics**Table**

S3. Pathogenicity test of *Diaporthe* spp. on hazelnut nuts (*Corylus avellana*) and their disease severity

Sr. no.	Isolates code	Fungal spp.	DS% [1]	SD [2]	TK [3]
1	Hwa-18-1	*Diaporthe eres*	86.1	4.81	efg
2	HsN5a-2	*D. eres*	80.6	9.62	defg
3	HsM2a-3	*D. eres*	77.8	4.81	defg
4	HMb-1b-1	*D. eres*	83.3	8.33	efg
5	HMb-5a-3	*D. eres*	100.0	0.00	g
6	Hma-10a-1	*D. eres*	80.6	4.81	defg
7	HM-2a-1	*D. eres*	75.0	8.33	cdefg
8	HM-4b-2	*D. eres*	91.7	8.33	fg
9	HM-5a-1	*D. eres*	80.6	9.62	defg
10	HM-8b-1	*D. eres*	83.3	8.33	efg
11	HM-11b-2	*D. eres*	88.9	9.62	efg
12	Hm-12a-1	*D. novem*	77.8	9.62	defg
13	HM-17a-1	*D. eres*	97.2	4.81	fg
14	Hm-20a-2	*D. oncostoma*	36.1	4.81	abcd
15	HBR-2b-1	*D. eres*	52.8	4.81	bcdef
16	HBr-3a-2	*D. eres*	55.6	9.62	bcdefg
17	HBR-8a-1	*D. eres*	86.1	9.62	efg
18	HBr-8b-2	*D. rudis*	86.1	4.81	efg
19	HBr-9b-1	*D. ravennica*	13.9	9.62	ab
20	HBR-12b-1	*D. eres*	88.9	12.73	efg
21	HBr-13a-2	*D. foeniculina*	72.2	4.81	cdefg
22	HBR-15b-1	*D. eres*	88.9	9.62	efg

23	HBR-16a-1	*D. eres*	58.3	8.33	bcdefg
24	HN-27a-2	*D. eres*	86.1	9.62	efg
25	HBr-27a-2	*D. rudis*	13.9	4.81	ab
26	Hm-30-2	*D. sojae*	91.7	8.33	fg
27	HN-31a-2	*D. eres*	69.4	12.73	cdefg
28	HM-43b-1	*D. eres*	88.9	12.73	efg
29	Hm-46a-1	*D. foeniculina*	77.8	12.73	defg
30	HM-93a-1	*D. eres*	94.4	4.81	fg
31	HN-100a-2	*D. eres*	58.3	8.33	bcdefg
32	HM-108b-2	*D. eres*	58.3	8.33	bcdefg
33	HN-104a-2	*D. eres*	80.6	4.81	defg
34	Hm-110b-1	*D. rudis*	94.4	4.81	fg
35	HM-112a-1	*D. eres*	44.4	9.62	abcde
36	HM-115b-3	*D. eres*	86.1	12.73	efg
37	Hm-128a-2	*D. novem*	30.6	4.81	abc
38	Hm-129a-2	*D. novem*	75.0	0.00	cdefg
39	HBr-108b-2	*D. foeniculina*	100.0	0.00	g
40	HM-137a-1	*D. eres*	75.0	0.00	cdefg
41	Healthy Control		0.00	0.00	a

[1] Disease severity in percentage [2] standard deviation [3] Tukey test

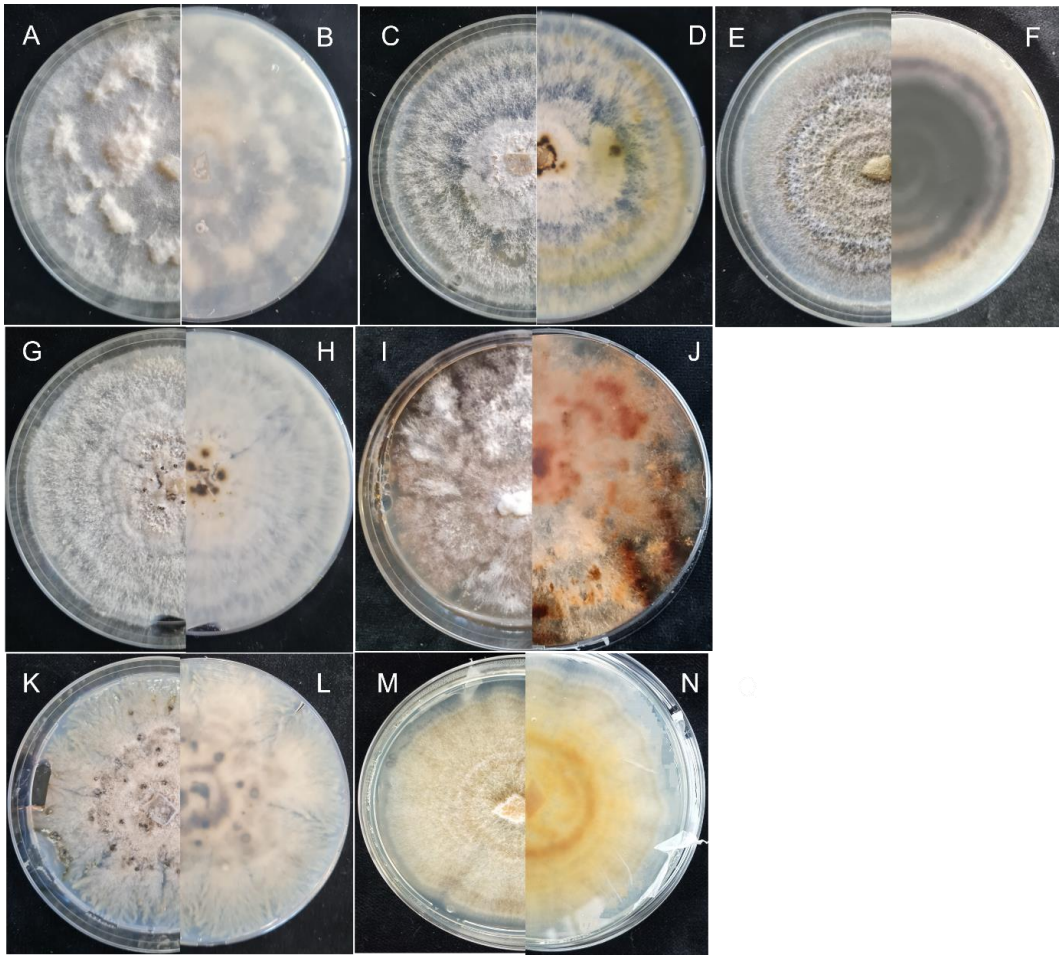


Fig. S1. Morphology of *Diaporthe* spp. obtained from hazelnut nuts **(A-B)** *D. sojae*. Front and reverse side of colony grown on PDA after 10 days at 23 ± 2 °C, **(C-D)** *D. foeniculina*. Front and reverse side of colony grown on PDA after 10 days at 23 ± 2 °C, **(E-F)** *D. novem*. Front and reverse side of colony grown on PDA after 10 days at 23 ± 2 °C, (**G-H)** *D. ravennica*. Front and reverse side of colony grown on PDA at 23 ± 2 °C, **(I-J)** *D. rudis*. Front and reverse side of colony grown on PDA after 10 days at 23 ± 2 °C, **(K-L)** *D. eres*. Front and reverse side of colony grown on PDA after 10 days at 23 ± 2 °C, (**M-N)** *D. oncostoma*. Front and reverse side of colony grown on PDA after 10 days at 23 ± 2 °C.

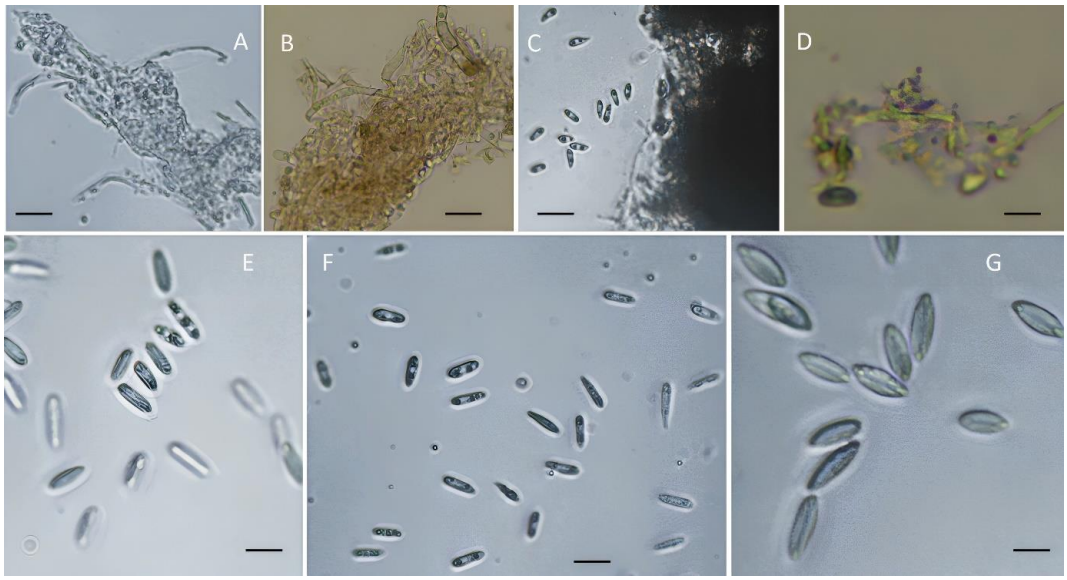


Fig. S2. Morphological features of *Diaporthe* spp. obtained from hazelnut nuts **(A)** Conidia of *D. sojae* (dimensions 5.8-7.3x2-3.2; scale bar: 10 µm), **(B)** Conidia of *D. foeniculina* (dimensions 7.6-9.5x2.5-2.9 µm; scale bar: 10 µm), **(C)** Conidia of *D. novem* (dimensions: 6.9-9.1x2.5-2.9 µm; scale bar: 10 µm), **(D)** Conidia of *D. ravennica* (dimensions 8.3-11.9x2.5-4.3 µm; scale bar: 10 µm), **(E)** Conidia of *D. rudis* (dimensions: 7.5-8.5x2.4-2.7 µm; scale bar: 10 µm), **(F)** Conidia of *D. eres* (dimensions: 8.5-11.1x 3.5-4.5 µm; scale bar: 10 µm), **(G)** Conidia of *D. oncostoma* (11-14.5x 3.2-4.9 µm; scale bar: 10 µm).

References

Aiello, D., Guarnaccia, V., Costanzo, M. B., Leonardi, G. R., Epifani, F., Perrone, G., and Polizzi, G. 2022. Woody Canker and Shoot Blight Caused by Botryosphaeriaceae and Diaporthaceae on Mango and Litchi in Italy. Horticulturae 8:330.

Annesi, T., Luongo, L., Vitale, S., Galli, M., and Belisario, A. 2016. Characterization and pathogenicity of *Phomopsis theicola* anamorph of *Diaporthe foeniculina* causing stem and shoot cankers on sweet chestnut in Italy. J. Phytopathol. 164: 412–416.

Arciuolo, R., Chiusa, G., Castello, G., Camardo Leggieri, M., Spigolon, N., and Battilani, P. 2022. *Diaporthe* spp. is confirmed as the main fungus associated with defective Turkish hazelnuts. Plant Health Prog. doi: 10.1094/PHP-01-22-0006-RS.

Arciuolo, R., Santos, C., Soares, C., Castello, G., Spigolon, N., Chiusa, G., Lima, N., and Battilani, P. 2020. Molecular characterization of *Diaporthe* species associated with hazelnut defects. Front. Plant Sci. 11:611655.

Bai, Y., Pan, M., Gao, H., Lin, L., Tian, C., and Fan, X. 2022. Studies of *Diaporthe* Species Causing Hazelnut Canker Disease in Beijing, China, with Two New Species Described. Plant Pathol. 00:1-12.

Battilani, P., Chiusa, G., Arciuolo, R., Somenzi, M., Fontana, M., Castello, G., and Spigolon, N. 2018. *Diaporthe* as the main cause of hazelnut defects in the Caucasus region. Phytopathol. Mediterr., 57:320-333.

Belisario, A., Maccaroni, M., Coramusi, A., Corazza, L., Pryor, B. M., and Figuli, P. 2004. First report of *Alternaria* species groups involved in disease complexes of hazelnut and walnut fruit. Plant Dis. 88:426-426.

Bertetti D., Guarnaccia V., Spadaro D., Gullino M.L. 2018. First Report of Fruit Rot in European Pear Caused by *Diaporthe eres* in Italy. Plant Dis. 102:2651.

Carbone, I., and Kohn, L. M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91:553–556.

Chaisiri, C., Liu, X., Lin, Y., Fu, Y., Zhu, F., and Luo, C. 2021. Phylogenetic and haplotype network analyses of *Diaporthe eres* species in China based on sequences of multiple loci. Biology 10:179.

Dai, D. Q., Wijayawardene N. N., Bhat, D. J., Chukeatirote, E., Bahkali, A. H., Zhao, R. L, Xu, J. C., and Hyde, K. D. 2014. *Pustulomyces* gen. nov. accommodated in Diaporthaceae, Diaporthales, as revealed by morphology and molecular analyses. Cryptogam. Mycol. 35:63-72.

Díaz, G. A., Latorre, B. A., Lolas, M., Ferrada, E., Naranjo, P., and Zoffoli, J. P. 2017. Identification and characterization of *Diaporthe ambigua*, *D. australafricana*, *D. novem*, and *D. rudis* causing a postharvest fruit rot in kiwifruit. Plant Dis. 101:1402-1410.

Dissanayake, A. J., Camporesi, E., Hyde, K. D., Wei, Z., Yan, J. Y., and Li, X. H. 2017 Molecular phylogenetic analysis reveals seven new *Diaporthe* species from Italy. Mycosphere 8:853–877.

Eichmeier, A., Pecenka, J., Spetik, M., Necas, T., Ondrasek, I., Armengol, J., León, M., Berlanas, C. and Gramaje, D. 2020. Fungal trunk pathogens associated with *Juglans regia* in the Czech Republic. Plant dis.104:761-771.

Elfar, K., Torres, R., Diaz, G. A., and Latorre, B. A. 2013. Characterization of *Diaporthe australafricana* and *Diaporthe* spp. associated with stem canker of blueberry in Chile. Plant Dis. 97:1042-1050.

Fu, C. H., Hsieh, H. M., Chen, C. Y., Chang, T. T., Huang, Y. M., Ju, Y. M. 2013. *OphioDiaporthe cyatheae* gen. et sp. nov., a diaporthalean pathogen causing a devastating wilt disease of *Cyathea lepifera* in Taiwan. Mycologia 105: 861-872.

Gajanayake, A. J., Abeywickrama, P. D., Jayawardena, R. S., Camporesi, E., and Bundhun, D. 2020. Pathogenic *Diaporthe* from Italy and the first report of

D. foeniculina associated with *Chenopodium* sp. Plant Pathol. Quar. 10:172-197.

Gao, Y. H., Liu, F., Duan, W., Crous, P. W., and Cai, L. 2017. *Diaporthe* is paraphyletic. IMA fungus 8:153-187.

Gao, H., Pan, M., Tian, C., and Fan, X. 2021. *Cytospora* and *Diaporthe* species associated with hazelnut canker and dieback in Beijing, China. Front. cell. infect. 11.

Glass, N. L., Donaldson, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Appl. Environ. Microbiol. 61:1323–1330.

Glei, M., Fischer, S., Lamberty, J., Ludwig, D., Lorkowski, S., and Schlörmann, W. 2018. Chemopreventive Potential of In Vitro Fermented Raw and Roasted Hazelnuts in LT97 Colon Adenoma Cells. Anticancer Res. 38:83-93.

Gomes, R. R., Glienke, C., Videira, S. I. R., Lombard, L., Groenewald, J. Z., and Crous, P. W. 2013. *Diaporthe*: a genus of endophytic, saprobic and plant pathogenic fungi. Persoonia: Molecular Phylogeny and Evolution of Fungi 31:1-41.

Guarnaccia, V., Martino, I., Tabone, G., Brondino, L., and Gullino, M. L. 2020. Fungal pathogens associated with stem blight and dieback of blueberry in northern Italy. Phytopathol. Mediterr. 59:229-245.

Guarnaccia, V., Groenewald, J. Z., Woodhall, J., Armengol, J., Cinelli, T., Eichmeier, A., Ezra, D., Fontaine, F., Gramaje, D., Gutierrez-Aguirregabiria, A., Kaliterna, J., Kiss, L., Larignon, P., Luque, J., Mugnai, L., Naor, V., Raposo, R., Sándor, E., Váczy, K.Z., and Crous, P. W. 2018. *Diaporthe* diversity and pathogenicity revealed from a broad survey of grapevine diseases in Europe. Persoonia: Molecular Phylogeny and Evolution of Fungi 40:135-153.

Guarnaccia, V., and Crous, P. W. 2018. Species of *Diaporthe* on Camellia and Citrus in the Azores Islands. Phytopathol. Mediterr. 57:307-319.

Guarnaccia, V., Vitale, A., Cirvilleri, G., Aiello, D., Susca, A., Epifani, F., Perrone, G. and Polizzi, G. 2016. Characterization and pathogenicity of fungal species associated with branch cankers and stem-end rot of avocado in Italy. Eur. J. Plant Pathol. 146:963-976.

Guerrero Contreras, J., Galdames Gutierrez, R., Ogass Contreras, K., Pe´rez Fuentealba, S. 2020. First report of *Diaporthe foeniculina* causing black tip and necrotic spot on hazelnut kernel in Chile. Plant dis. 104:975.

Hilário, S., Gonçalves, M. F., and Alves, A. 2021. Using genealogical concordance and coalescent-based species delimitation to assess species boundaries in the *Diaporthe eres* complex. Journal of Fungi 7:507.

Huang, F., Udayanga, D., Wang, X., Hou, X., Mei, X., Fu, Y., Hyde, K.D. and Li, H. 2015. Endophytic Diaporthe associated with Citrus: A phylogenetic reassessment with seven new species from China. Fungal Biol. 119:331-347.

Hyde, K. D., Dong, Y., Phookamsak, R., Jeewon, R., Bhat, D. J., Jones, E. B., Liu, N. G., Abeywickrama, P. D., Mapook, A., Wei, D., and Perera, R. H. 2020. Fungal diversity notes 115–1276: taxonomic and phylogenetic contributions on genera and species of fungal taxa. Fungal Diversity 100:5-277.

Hillis, D. M., and Bull, J. J., 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Systematic Biology 42:182-192.

ISTAT. 2021. http://dati.istat.it/Index.aspx?QueryId=33702. Accessed 13 September 2022.

Katoh, K., and Standley, D. M. 2013. MAFFT Multiple sequence alignment software version 7: Improvements in performance and usability. Mol. Biol. Evol. 30:772-780.

Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33:1870-1874.

Lamprecht, S. C., Crous, P. W., Groenewald, J. Z., Tewoldemedhin, Y. T., Marasas, W. F. O., 2011. Diaporthaceae associated with root and crown rot of maize. IMA Fungus 2:13-24.

Lawrence, D. P., Travadon, R., and Baumgartner, K. 2015. Diversity of *Diaporthe* species associated with wood cankers of fruit and nut crops in northern California. Mycologia 107: 926-940.

León, M., Berbegal, M., Rodríguez-Reina, J. M., Elena, G., Abad-Campos, P., Ramón-Albalat, A., Olmo, D., Vicent, A., Luque, J., Miarnau, X. and Agustí-Brisach, C. 2020. Identification and characterization of *Diaporthe* spp. associated with twig cankers and shoot blight of almonds in Spain. Agronomy 10:1062.

Lesuthu, P., Mostert, L., Spies, C. F. J., Moyo, P., Regnier, T., and Halleen, F. 2019. *Diaporthe nebulae* sp. nov. and first report of *D. cynaroidis*, *D. novem*, and *D. serafiniae* on grapevines in South Africa. Plant Dis. 103:808-817.

Librandi, I., Galli M., and Belisario, A. 2006. Le patologie del frutto del nocciolo in Italia, con particolare riguardo alla zona del viterbese. Petria 16:125–134.

Linaldeddu, B. T., Deidda, A., Scanu, B., Franceschini, A., Alves, A., Abdollahzadeh, J., and Phillips, A. J. L. 2016. Phylogeny, morphology and pathogenicity of Botryosphaeriaceae, Diatrypaceae and Gnomoniaceae associated with branch diseases of hazelnut in Sardinia (Italy). Eur. J. Plant Pathol. 146:259-279.

Lombard, L., van Leeuwen, G., Guarnaccia, V., Polizzi, G., van Rijswick, P., Rosendahl, K., Gabler, J. and Crous, P. 2014. *Diaporthe* species associated with Vaccinium, with specific reference to Europe. Phytopathol. Mediterr. 53:287-299.

Luna, I. J., Besoain, X., Saa, S., Peach-Fine, E., Morales, F. C., Riquelme, N., Larach, A., Morales, J., Ezcurra, E., Ashworth, V. E. and Rolshausen, P. E. 2022. Identity and pathogenicity of Botryosphaeriaceae and Diaporthaceae from *Juglans regia* in Chile. Phytopathol. Mediterr. 61:79-94.

Marin-Felix, Y., Hernández-Restrepo, M., Wingfeld, M. J., Akulov, A., Carnegie, A. J., Cheewangkoon, R., Gramaje, D., Groenewald, J. Z., Guarnaccia, V., Halleen, F., Lombard, L., Luangsaard, J., Marincowitz, S., Moslemi, A., Mostert, L., Quaedvlieg, W., Schumacher, R. K., Spies, C. F. J., Tangavel, R., Taylor, P. W. J., Wilson, A. M., Wingfeld, B. D., Wood, A. R., and Crous, P. W. 2019. Genera of phytopathogenic fungi: GOPHY 2. Stud. Mycol. 92:47-133.

Moral, J., Agustí-Brisach, C., Pérez-Rodríguez, M., Xavier, C., Raya, M. C., Rhouma, A., and Trapero, A. 2017. Identification of fungal species associated with branch dieback of olive and resistance of table cultivars to *Neofusicoccum mediterraneum* and *Botryosphaeria dothidea*. Plant Dis. 101:306-316.

Nitschke, T. 1870. *Pyrenomycetes Germanici*. 2:245 Breslau. Eduard Trewendt, Germany**.**

Nunzio, M. D. 2019. Hazelnuts as source of bioactive compounds and health value underestimated food. Curr. Res. Nutr. Food Sci. 7:17-28.

Nylander, J. A. A. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.

O'Donnell, K., and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylogenet. Evol. 7:103-116.

Paradinas, A., Ramade, L., Mulot-Greffeuille, C., Hamidi, R., Thomas, M. Toillon, J. 2022. Phenological growth stages of 'Barcelona' hazelnut (*Corylus*

avellana L.) described using an extended BBCH scale. Sci. Hortic. 296:110902.

Phukhamsakda, C., McKenzie, E. H., Phillips, A. J., Gareth Jones, E. B., Jayarama Bhat, D., Stadler, M., ... and Hyde, K. D. 2020. Microfungi associated with *Clematis* (Ranunculaceae) with an integrated approach to delimiting species boundaries. Fungal diversity 102:1-203.

Prencipe, S., Nari, L., Vittone, G., Spadaro, D. (2017) First Report of *Diaporthe eres* causing stem canker on peach (*Prunus persica*) in Italy. Plant Dis. 101:1052.

Pscheidt, J. W., Heckert, S., Wiseman, M., and Jones, L. 2019. Fungi associated with and influence of moisture on development of kernel mold of hazelnut. Plant Dis. 103:922-928.

Rayner, R. W. 1970. A mycological colour chart. Commonwealth Mycological Institute, Kew.

Ronquist, F., Teslenko, M., van der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M. A., Huelsenbeck, J. P. 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61:539-542.

Santos, L., Alves, A., Alves, R. 2017. Evaluating multi-locus phylogenies for species boundaries determination in the genus *Diaporthe*. PeerJ, 5:e3120.

Santos, J. M., Correia, V. G., and Phillips, A. J. 2010. Primers for mating-type diagnosis in *Diaporthe* and *Phomopsis*: their use in teleomorph induction in vitro and biological species definition. Fungal Biol. 114:255-270.

Scarpari, M., Vitale, S., Di Giambattista, G., Luongo, L., De Gregorio, T., Schreiber, G., ... and Voglmayr, H. 2020. *Didymella corylicola* sp. nov., a new fungus associated with hazelnut fruit development in Italy. Mycol. Prog. 19:317-328.

Senanayake, I. C. Crous, P. W. Groenewald, J. Z. Maharachchikumbura, S. S. N. Jeewon, R. Phillips, A. J. L. Bhat, J. D. Perera, R. H. Li, Q. R. Li, W. J., ... and Hyde, K. D. 2017. Families of Diaporthales based on morphological and phylogenetic evidence. Stud. Mycol. 86:217-296.

Silvestri, C., Bacchetta, L., Bellincontro, A., and Cristofori, V. 2021. Advances in cultivar choice, hazelnut orchard management, and nut storage to enhance product quality and safety: an overview. J. Sci. Food Agric. 101:27-43.

Slippers, B., Crous, P. W., Jami, F., Groenewald, J. Z., and Wingfield, M. J. 2017. Diversity in the Botryosphaeriales: Looking back, looking forward. Fungal Biol. 121:307-321.

Smith, H., Wingfeld, M, J., Coutinho, T. A., and Crous, P. W. 1996. *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. S. Afr. J. Bot. 62:86-88.

Spadaro, D., Meloni, G.R., Siciliano, I., Prencipe, S., and Gullino, M.L. 2020. HPLC-MS/MS method for the detection of selected toxic metabolites produced by *Penicillium* spp. in nuts. Toxins 12: 307.

Swofford, D. L. 2003. PAUP* Phylogenetic Analysis Using Parsimony, (*and Other Methods); Version 4.0 b10; Sinauer Associates: Sunderland, MA, USA.

Teviotdale, L., Michailides, T. J., and Pscheidt, J. W. 2002. Compendium of Nut Crop Diseases in Temperate Zones. American Phytopathological Society, St Paul, MN, USA, pp 100.

Thambugala KM, Daranagama DA, Phillips AJL, Bulgakov TS et al. 2017 – Microfungi on Tamarix. Fungal Diversity 82:239–306.

Thompson, S. M., Tan, Y. P., Young, A. J., Neate, S. M., Aitken, E. A. B., and Shivas, R. G. 2011. Stem cankers on sunflower Helianthus annuus in Australia reveal a complex of pathogenic *Diaporthe* (*Phomopsis*) species. Persoonia 27:80–89.

Udayanga, D., Castlebury, L. A., Rossman, A. Y., Chukeatirote, E., and Hyde, K. D. 2015. The *Diaporthe* s*ojae* species complex: phylogenetic re-assessment of pathogens associated with soybean, cucurbits and other field crops. Fungal. Biol. 119:383-407.

Udayanga, D., Castlebury, L. A., Rossman, A. Y., Chukeatirote, E., and Hyde, K. D. 2014. Insights into the genus *Diaporthe*: phylogenetic species delimitation in the *D. eres* species complex. Fungal Diversity 67:203-229.

Udayanga, D., Liu, X., Crous, P. W., McKenzie, E. H. C., Chukeatirote, E., and Hyde, K. D. 2012. A multi-locus phylogenetic evaluation of *Diaporthe* (Phomopsis). Fungal Diversity 56:157-171.

Udayanga, D., Liu, X., McKenzie, E. H., Chukeatirote, E., Bahkali, A. H., and Hyde, K. D. 2011. The genus *Phomopsis*: biology, applications, species concepts and names of common phytopathogens. Fungal Diversity 50:189-225.

Valente, S., Meloni, G.R., Prencipe, S., Spigolon, N., Somenzi, M., Fontana, M., Gullino, M.L., and Spadaro, D. 2020. Effect of drying temperature and exposure times on *Aspergillus flavus* growth and aflatoxin production on artificially inoculated hazelnuts. J. Food Prot. 83:1241-1247.

Vitale, S. M., Scarpari, L., Luongo, M., Galli, A., Belisario, G., Schreiber, M., Petrucci, G., Fontaniello, T., De Gregorio, G., Castello. 2020. Nocciola avariata, un fenomeno in aumento che abbatte la qualità**.** Rivista di Frutticoltura, published online (available at: https://rivistafrutticoltura.edagricole.it/post-raccolta/nocciola-avariata-un-fenomeno-in-aumento-che-abbatte-la-qualita/)

Waqas, M., Guarnaccia, V., and Spadaro, D. 2022. First Report of Nut Rot Caused by *Neofusicoccum parvum* on Hazelnut (Corylus avellana) in Italy. Plant Dis. 106:1987.

Wehmeyer, L. E. 1926. A biologic and phylogenetic study of stromatic Sphaeriales. Am. J. Bot. 13:575-645.

White. T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: a guide to methods and applications. (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). Academic Press, New York, USA. 315–322.

Wiman, N. G., Webber III, J. B., Wiseman, M., and Merlet, L. 2019. Identity and pathogenicity of some fungi associated with hazelnut (*Corylus avellana* L.) trunk cankers in Oregon. Plos one 14:e0223500.

Yang, Q., Fan, X. L., Guarnaccia, V., and Tian, C. M. 2018. High diversity of *Diaporthe* species associated with twelve new species described. MycoKeys 39:97-149.

Yang, Q., Jiang, N., and Tian, C. M. 2020. Three new *Diaporthe* species from Shaanxi province, China. MycoKeys 67:1.

Yang, Q., Jiang, N., and Tian, C. M. 2020. Three new *Diaporthe* species from Shaanxi province, China. MycoKeys, 67:1.

Yang, Q., Jiang, N., and Tian, C. M. 2021. New species and records of *Diaporthe* from Jiangxi Province, China. MycoKeys, 77:41.

Zapata, M., Palma, M. A., Aninat, M. J., and Piontelli, E. 2020. Polyphasic studies of new species of *Diaporthe* from native forest in Chile, with descriptions of *Diaporthe araucanorum* sp. nov., *Diaporthe foikelaweni* sp. nov. and *Diaporthe patagonica* sp. nov. Int. J. Syst. Evol. Microbiol. 70:3379-3390.

Chapter 4

plant disease

First report of nut rot caused by *Neofusicoccum parvum* on hazelnut (*Corylus avellana*) in Italy

M. Waqas[1], V. Guarnaccia[1,2], D. Spadaro[1,2]†

[1] Department of Agricultural, Forest and Food Sciences, University of Torino, I-10095 Grugliasco, TO, Italy.

[2] Centre of Competence for the Innovation in the Agro-environmental Sector, AGROINNOVA, University of Torino, I-10095 Grugliasco, TO, Italy.

†Corresponding author: Davide Spadaro; davide.spadaro@unito.it

Published in Disease Note (2022), https://doi.org/10.1094/PDIS-10-21-2249-PDN

Hazelnut (*Corylus avellana* L.), which is native to Europe and Western Asia, is a widely distributed and economically important crop in Italy; it is cultivated on 82,104 ha and its production is 110,618 t (ISTAT 2021). A total of 111 black rotted nuts (incidence: 41%) with sunken lesions from Lu and Cuccaro (45°00′21.8″N 8°28′59.6″E), northwestern Italy were collected during the ripening stage of hazelnuts from October to December 2020. Symptomatic half-cut kernels were sterilized in 1% NaClO for 1 min, washed in sterile water twice, and dried on sterile filter paper. The fragments were placed onto potato dextrose agar (PDA) containing streptomycin. After 48 to 72 h of incubation at 25°C, fast-growing white colonies with abundant aerial mycelia were observed. On the opposite side of the plates, colonies were initially white and then turned to dark-grayish olive after one week of incubation. Dark colonies produced globose, hyaline, ellipsoidal, and unicellular conidia ranging from 12.23 to 15 × 5 to 6.71 μm. Morphologically, the causal agent was identified as *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (Crous et al. 2006; Zhang et al. 2021). The DNA from the isolates HMa-19-2 and Hwb-4b-2 was extracted with the E.Z.N.A. Fungal DNA mini kit (Omega Bio-Tek) according to manufacturer instructions. Molecular identification was confirmed by sequencing the rDNA internal transcribed spacer (ITS) using primers ITS1/ITS4 (White et al. 1990) and the translation elongation factor 1-alpha (*tef-1α*) gene using primers EF1-728F/EF1-986R (Carbone and Kohn 1999). The sequences of both isolates were deposited in GenBank for the ITS (accession nos. MZ848132 and MZ848133) and the *tef-1α* gene (accession nos. MZ913266 and MZ913267). BLAST analysis showed 99% identity with the ex-type strain of *N. parvum* (CMW9081) for ITS and *tef-1α*. The maximum likelihood method based on combined sequences of ITS and *tef-1α* genes was performed, and the isolates of *N. parvum* clustered with the ex-type strain of *N. parvum* (CMW9081). The pathogenicity of both isolates was tested on ripening hazelnuts (BBCH: 85) to evaluate Koch's postulates. Three nuts per isolate and per three replicates were

surface disinfected with 1% NaClO. A piece of shell (5 mm in diameter) from nuts was removed with a sterile cork borer, and nuts were inoculated with PDA mycelium plugs of the same diameter cut from 7-day-old PDA colonies (Seddaiu et al. 2021). The control nuts were treated with sterilized PDA plugs. All inoculated nuts produced black lesions with softening pulp. Additionally, abundant white-gray mycelium developed on the inoculation sites. Control nuts showed no symptoms. *N. parvum* was recently reported in Italy on chestnut (Seddaiu et al. 2021) and blueberry (Guarnaccia et al. 2021). The fungal pathogen was also reported as an agent of gray necrosis of hazelnut in Chile (Duran et al. 2020). However, this is the first report of *N. parvum* on hazelnut in Italy. The findings suggest that *N. parvum* could severely affect hazelnut production in Italy. Accurate identification of the pathogen will support growers in managing the disease.

References

Carbone, I., and Kohn, L. M. 1999. Mycologia 91:553

Crous, P.W., et al. 2006. Stud. Mycol. 55:235.

Duran, P., et al. 2020. New Disease Rep. 42:7.

Guarnaccia, V., et al. Phytopathol. Mediterr. 59:229.

ISTAT. 2021. http://dati.istat.it/Index.aspx?QueryId=33702 (accessed 26 August 2021).

Seddaiu, S., et al. 2021. Plant Dis. 205: 3733.

White, T. J., et al. 1990. Page 315 in: PCR Protocols: a Guide to Methods and Applications. Academic Press, San Diego, CA.

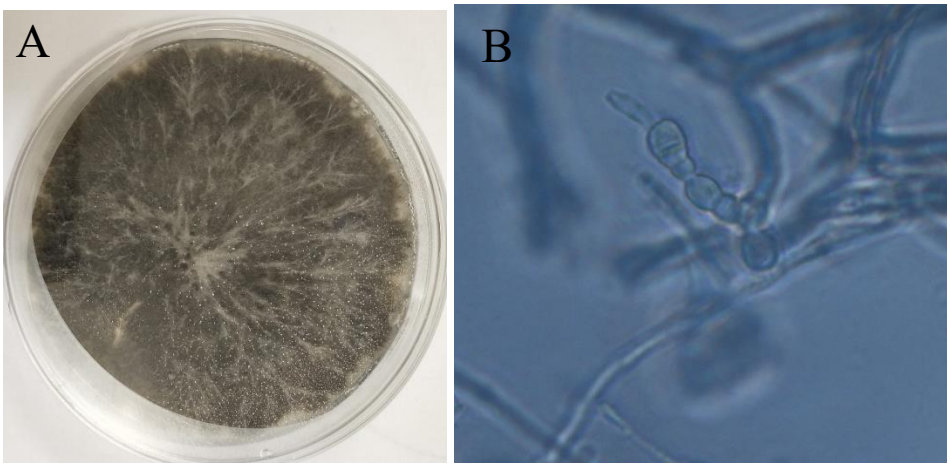
Zhang, W., et al. 2021. Persoonia 46:63.

The author(s) declare no conflict of interest.

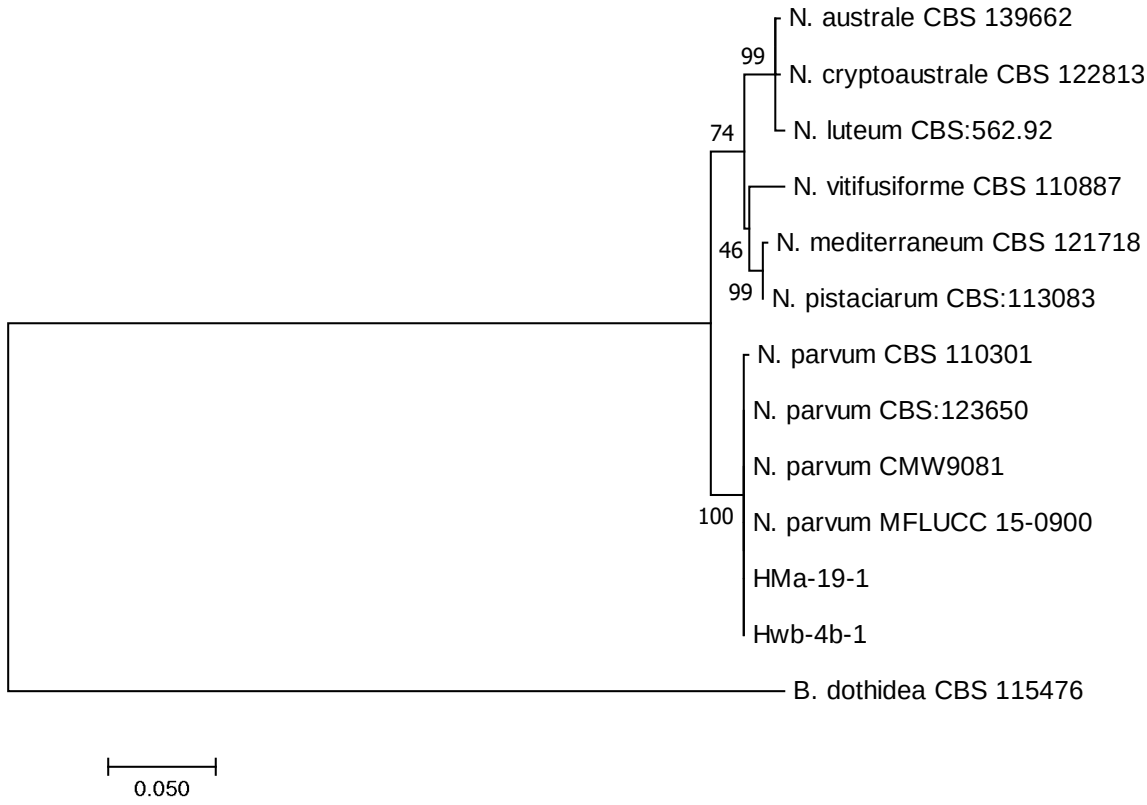
Keywords

nut rot, hazelnut, *Neofusicoccum parvum*, Italy

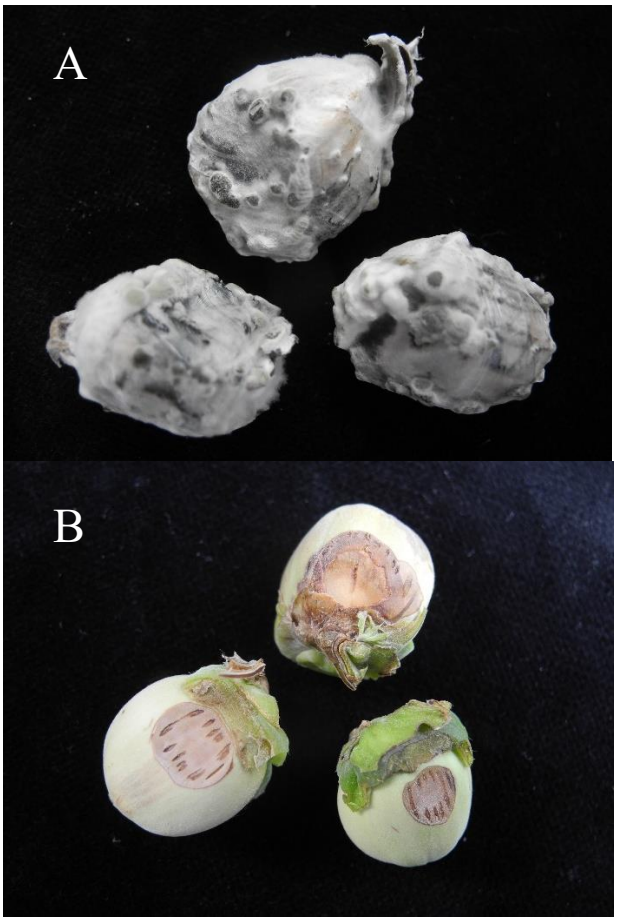
e-Xtra Files



Supplementary Figure 1. *N. parvum* (A) Colony on PDA (B) Conidiogenous cells



Supplementary Figure 2. Maximum likelihood phylogenetic tree based on the concatenated ITS region and *tef-1α* gene sequence datasets. Evolutionary analysis was conducted using MEGA, version 7. The tree was rooted with *B. dothidea* (CBS115476).



Supplementary Figure 3. (A) Black lesions and white-grey mycelia on hazelnuts after inoculation with *N. parvum* (B). Healthy hazelnut kernel after inoculation with sterilized PDA plug.

Chapter 5

Host range and phylogenesis of the four races of *Fusarium oxysporum* f. sp. *lactucae*

Muhammad Waqas[1], Giovanna Gilardi[2], Vladimiro Guarnaccia[1,2], Angelo Garibaldi[2], Davide Spadaro[1,2,†]

[1] Dept. Agricultural, Forest and Food Sciences (DISAFA), University of Torino, Grugliasco (TO), Italy

[2] Centre of Competence for the Innovation in the Agro-environmental Sector (AGROINNOVA), University of Torino, Grugliasco (TO), Italy

†Corresponding author: D. Spadaro; davide.spadaro@unito.it

Keywords

forma specialis; *Fusarium oxysporum*; *Lactuca sativa*; host range; molecular identification; race; species complex; vascular wilt.

Abstract

Vascular wilt of lettuce is caused by strains of *Fusarium oxysporum* species complex. The most common symptoms of Fusarium wilt on lettuce are reddish-brown discoloration of the vascular vessels, leaf yellowing with necrosis, stunted growth, wilting and plant death. According to their biological activity, these strains are considered as members of the *forma specialis lactucae* (FOL). The aim of this study was to characterize strains of *Fusarium oxysporum*, isolated from lettuce in Italy and other countries, belonging to the four currently known races, based on pathogenicity on different hosts and on combined phylogenetic analysis of *rpb2*, *tef-1α*, *cmdA* and *tub2*. For some uncharacterized strains, the race was determined through pathogenicity test with a set of differential lettuce cultivars, showing they belong to race 1 and race 4. Strain FOL 1/16 was assigned to race 4, therefore, this study established that the first occurrence of FOL race 4 in Italy to 2016. Pathogenicity was performed on five species to assess the host range: lettuce (*Lactuca sativa*), lamb's lettuce (*Valerianella olitoria*), spinach (*Spinacia oleracea*), wild rocket (*Diplotaxis tenuifolia*) and cultivated rocket (*Eruca sativa*). Most FOL strains were pathogenic on lettuce with different levels of disease severity; in particular, FOL races 3 and 4 showed the highest level of virulence. FOL races 3 and 4 were pathogenic on lettuce and lamb's lettuce. Results of multigene phylogenetic analysis showed that FOL races 1 and 4 belong to *F. curvatum*, strains of race 2 to *F. curvatum* and *F. odoratissimum* and strains of race 3 to *F. cugenangense*. Phylogenetically, race 4 is very close to race 1, but by considering the host spectrum, it showed more similarities to race 3. This study shows for the first time a broader host range for the races 3 and 4 of FOL than previously reported and it demonstrates that phylogenetic analysis shows the separation of some races of FOL, but alone it is inconclusive, therefore it should be accompanied by biological assays based on pathogenicity.

Key words: *forma specialis*; *Fusarium oxysporum*; *Lactuca sativa*; host range; molecular identification; race; species complex; vascular wilt.

Introduction

Lettuce (*Lactuca sativa* L.), belonging to the Asteraceae, is one of the most economically important vegetables worldwide. Lettuce is a main component of salads and a great source of calcium, fiber, potassium, protein, carotenoids, antioxidants, vitamins, and minerals (Llorach et al. 2008). In Italy, lettuce is mainly cultivated in open fields on 12,167 ha with a production of about 278,000 t, and in greenhouses on 5,528 ha with a production of about 202,000 t (ISTAT 2022).

Lettuce production can be affected by several diseases, among them Fusarium wilt of lettuce, an economically important soilborne disease caused by *Fusarium oxysporum* f. sp. *lactucae* (FOL) (Gullino et al. 2019; Subbarao et al. 2017). FOL colonizes the xylem tissues of lettuce, causing disruption of water movement. As the disease progresses, leaves become yellow with necrosis, severe wilting and stunting that can lead to plant death. The vascular system of infected lettuce turns reddish brown and eventually decays (Gordon and Koike 2015; Subbarao et al. 2017). Fusarium wilt was first observed by Motohashi et al. (1960) in Japan and later the agent was identified as the forma specialis *lactucae* of *F. oxysporum* (Matuo and Motohashi, 1967). Fusarium wilt was later reported in the USA (Hubbard and Gerik 1993) and Iran (Millani et al. 1999). FOL was absent in Europe until 2002, when lettuce plants infected by FOL were reported in Italy (Garibaldi et al. 2002). The seed trade provides an inadvertent source of pathogen dissemination between countries and the rapid spread of Fusarium wilt of lettuce due to FOL contaminated seeds (Garibaldi et al. 2004a; Franco Ortega et al. 2018).

FOL is categorized into four races (race 1, 2, 3 and 4) based on virulence patterns on resistant or susceptible varieties of lettuce, with different worldwide distributions. FOL race 1 was first reported in Japan in 1967 (Matuo and Motohashi

1967), then in the USA (Hubbard and Gerik 1993), Iran (Millani et al. 1999), Taiwan (Huang and Lo 1998), Brazil (Ventura and Costa 2008), Italy (Garibaldi et al. 2002), Portugal (Marques Ramalhete et al. 2006), Spain (Guerrero et al. 2020), Norway (Herrero et al. 2021), Greece (Tziros and Karaoglanidis 2023) and the UK (van Amsterdam et al. 2023). FOL races 2 and 3 were reported in Japan (Fujinaga et al., 2001; 2005) and FOL race 3 was reported in Taiwan (Lin et al. 2014). Race 4, the last race in order of appearance) was reported in the Netherlands (Gilardi et al. 2017b), Belgium (Claerbout et al. 2018), Italy (Gilardi et al. 2019), the UK (Taylor et al. 2019), and Spain (Galvez et al. 2023).

Until the early 21st century, *Fusarium oxysporum* species complex (FOSC) were identified based on morphological characteristics but phenotypic characters can be challenging or controversial, even for trained mycologists (Leslie and Summerell 2006). To address these issues, multiple gene markers were developed for taxonomic and identification purposes in *Fusarium* spp. (Geiser et al. 2004). Translation elongation factor 1-alpha (*tef-1α*), RNA polymerase II subunits (RPB1 and *rpb2*), the Large Subunit (LSU) of the nuclear ribosomal RNA, polygalacturonase genes, and the intergenic spacer region (IGS) of the ribosomal operon have been useful target regions to differentiate the formae speciales (Geiser et al. 2013; Mbofung et al. 2007; O'Donnell et al. 2015). In particular, *tef-1α* and *rpb2* have been extensively used for identification as they provide good resolution power and increased discrimination in *Fusarium* species (O'Donnell et al. 2000, 2009). Beta tubulin 2 (*tub2*) and calmodulin (*cmdA*) genes were also used along with *tef-1α* and *rpb2* at species level (Lombard et al. 2019; Claerbout et al. 2023). Currently, *F. oxysporum* is considered a species complex consisting of numerous cryptic species. Recently, *F. oxysporum* sensu stricto has been epitypified to allow for the formal naming of 15 cryptic taxa in the species complex (Lombard et al. 2019).

In this work, we aimed to evaluate the pathogenicity of *F. oxysporum* strains isolated from lettuce on leafy vegetables, including lettuce. To this aim, we first determined the race for some strains of *F. oxysporum* f. sp. *lactucae*. A second aim was to establish the new species name according to Lombard et al. (2019) for the four races of *F. oxysporum* f. sp. *lactucae*, based on phylogenetic analyses. The physiological race, the host range and the species assigned based on the recent taxonomy were discussed.

Materials and Methods

Fungal strains

Eighteen monoconidial strains of *Fusarium oxysporum*, isolated from lettuce, were retrieved from the culture collection of Agroinnova, University of Turin (Italy), where they were stored at -80°C in 20 % glycerol. They were selected based on pathogenicity and preliminary analysis of *rpb2*, and *tef-1α* (**Table 1**). All Italian FOL strains were obtained from diseased lettuce plants grown in farms from Southern and Northern Italy. Japanese and Taiwanese strains were kindly provided by Dr. M. Fujinaga and Dr. J. H. Huang, respectively. American strains AZ-01 and JCP 0043 were kindly provided by Dr. J. Petersen and Dr. M. E. Matheron, respectively. Belgian and Dutch strains were kindly provided by Monica Höfte and Johan Meffert, respectively.

Pathogenicity test

Fusarium strains were tested for pathogenicity on five plant species belonging to different botanical families: lettuce [*Lactuca sativa* (cv. Romana Verde, ISI Sementi, Fidenza, Italy)]; wild rocket [*Diplotaxis tenuifolia* (cv. Winter, Orosem, Azzano San Paolo, Italy)]; cultivated rocket [*Eruca sativa* (cv. Coltivata, Furia Sementi, Parma, Italy)]; lamb's lettuce [*Valerianella olitoria* (cv. Trophy, RB

Sementi, Torino, Italy)]; and spinach [*Spinacia oleracea* (cv. Zebu, Rijk Zwaan, Bologna, Italy)]. Plants were grown in a glasshouse at 23 to 25°C for 15 days before transplanting and inoculation. The inoculum was prepared by growing the fungus in 1 L Erlenmeyer flasks (Bibby Sterilin Ltd., Stone, UK) containing 150 ml of Potato Dextrose Broth (PDB, Merck, Darmstadt, Germany) by shaking (acceleration, 0.1 m^2/s) at 25°C under constant light. After 10 days, cultures were filtered through sterilized double-layer of cheesecloth to collect conidia, which were adjusted to a final concentration of 10^6 conidia/ml with sterile deionized water. Seedlings (15-to-20-days old) were removed from the substrate (peat: perlite 70:30 v/v) and immediately inoculated with the fungal strains by dipping the roots into 100 mL of conidial suspension for 5 min. Inoculated seedlings were transplanted into 12 L pots, filled with a peat mixture (previously steamed at 70 °C for 1 h) with 7 plants/pot (experimental unit), with three replicates thus resulting in a total of 28 plants per isolate per trial. Control plants were prepared similarly and soaked in sterilized water. The pots were then placed in a greenhouse at air temperatures ranging from 28 to 32°C. A randomized complete block design was adopted. The test was performed 3 times.

Five FOL strains shown in **Table S1** were tested for pathogenicity on four differential lettuce varieties [Romana Romabella and Costa Rica No. 4 resistant to race 1, Cencibel (Rijk Zwaan), and Kiribati (Rijk Zwaan) resistant to race 4] for the identification of the physiological races of *F. oxysporum* f. sp. *lactucae* (Gilardi et al. 2017b; Tziros and Karaoglanidis 2023). The same experimental design as described above except with two repetitions was adopted.

Disease severity (DS) was assessed 14, 21, 28, and 35 days after inoculation (DAI) by using a score ranged from 0 to 4 (Gilardi et al. 2017b): where: 0= healthy plant/no disease; 1= initial symptoms of leaf chlorosis; 2= clear vascular browning, reduction in plant growth, and severe leaf chlorosis; 3=plant stunting and severe wilting with clear vascular browning and leaf chlorosis; and 4=dead plants/totally wilted. All the

data were expressed as disease index (DI) with a scale of 0-100, which was calculated using the formula:

DI = [∑ (i × ni)] / (4 × total of plants) × 100

with i = 0–4 and ni = the number of plants with rating i (Gilardi et al. 2017b). Additionally, based on the 0–100 disease index (DI) scale, the evaluated strains were classified with respect to their resistance or susceptibility as follows: 0-10= resistant; 11-30= partially resistant; 31–60= susceptible; and 61–100= highly susceptible (Tziros and Karaoglanidis 2023). The area under the disease progress curve (AUDPC) was calculated based on weekly collected data on disease severity and estimated according to the equation proposed by Shaner and Finney (1977).

$$\text{AUDPC} = \sum_{i=1}^{ni-1} [(yi + 1 + yi)\ /2][ti + 1 - ti]$$

Where *n*: total number of observations, *yi*: measure of the disease severity at *i*th observation, *ti*: the time interval (days) after observation of the disease at *i*th day.

DS and the AUDPC data for each strain and host obtained from the trials were analyzed by using analysis of variance (ANOVA). The mean values were separated by Tukey HSD ($P< 0.05$), by using SPSS software (IBM SPSS Statistics v. 28.0.1.0).

DNA extraction, PCR amplification, and sequencing

Genomic DNA of fungal cultures was extracted with an E.Z.N.A. Fungal DNA mini kit (Omega Bio-tek, Darmstadt, Germany) from 100 mg of mycelium grown for 7 days on Potato Dextrose Agar (PDA, VWR International, Leuven, Belgium) according to the manufacturer's procedure. The quality and concentration of extracted DNA were determined using NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The primers RPB2-5f2/RPB2-7cr (Liu et al. 1999; Sung et al. 2007) were used to amplify the RNA polymerase second largest subunit gene region (*rpb2*). Partial sequence of translation elongation factor 1-α (*tef-1α*) was

amplified by using EF-1/EF-2 primers (O'Donnell et al. 1998). The primers Cal228F/CAL2Rd (Carbone and Kohn 2019; Groenewald et al. 2013) were used to amplify part of a calmodulin gene (*cmdA*). While part of a beta-tubulin (*tub2*) gene was amplified with T1/4Rd (O'Donnell and Cigelnik 1997; Woudenberg et al. 2009). PCR mixtures and thermal cycling programs were adopted according to O'Donnell et al. (2008) for *rpb2*, Lombard et al. (2015) for *tef-1α* and Torbati et al. (2019) for *cmdA* and *tub2*. A volume of 5 µL PCR product for each PCR reaction was examined by electrophoresis at 100V on 1% agarose gel (VWR Life Science, AMRESCO® Biochemicals) stained with GelRed™. PCR products were sequenced by Eurofins Genomics Service (Milan, Italy).

Phylogenetic analysis

The DNA sequences obtained in this study were analyzed, and consensus sequences were computed using the Geneious software v.11.1.5 (Auckland, New Zealand), and compared to representative sequences from previous studies (Lombard et al. 2019). Sequences obtained from this study and sequences downloaded from GenBank, were aligned by using MAFFT v. 7.110 (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley 2013), and manually corrected, if necessary, with MEGA v. 7 (Kumar et al. 2016). The individual sequences generated in this study were compared with those maintained in the *Fusarium* MLST database and the NCBI's GenBank, and relevant sequences were included in the subsequent phylogenetic inference. Initially, phylogenetic analyses were performed individually for each gene based on Maximum Likelihood (ML) by using MEGA v. 7 and then combined in a multilocus analysis (*rpb2*, *tef-1α*, *cmdA* and *tub2*). The phylogeny was based on Bayesian Inference (BI) and Maximum Parsimony (MP) for the multi-locus analysis. For BI, the best evolutionary model for each locus was determined using MrModeltest v. 2.3 (Nylander 2004) and incorporated 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 at 0.2 and trees were sampled every 1,000 generations. The analysis stopped when the average standard deviation of split frequencies was below 0.01. The MP analysis was performed using Phylogenetic Analysis Using Parsimony (PAUP) 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', with all characters equally weighted 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 bootstrap analysis (Hillis and Bull 1993) were based on 1,000 replications. Sequences generated in this study were deposited in GenBank (**Table S2**).

Results

Fungal isolates and pathogenicity

Starting from a wider number of strains of *Fusarium oxysporum* isolated in Italy from different leafy vegetables present in the culture collection of Agroinnova (University of Torino), 18 strains belonging to four races of FOL were selected. This selection was based on their geographical origins by selecting reference isolates shared between different researchers, and based on preliminary pathogenicity and analysis of *rpb2* and *tef-1a* for further study (**Table 1**).

The pathogenicity test was conducted on differential cultivars to assign the correct race within the forma specialis *lactucae* to some of the strains of *F. oxysporum* isolated from lettuce. Results of pathogenicity for race determination showed that two strains (Fus. Lat. 4/15 and Fus. Lat. 3/18) and FOL 1/16 were assigned to race

1 and race 4 respectively. Race 1 strains were highly virulent on Kiribati and Cencibel, and race 4 strains were not. Race 1 strains showed disease severity more than 90% while the disease severity of race 4 strains ranged from 58% to 92%. Race 4 strains were highly virulent on Costa Rica No. 4 and Romana Romabella and race 1 strains were not. (**Table S1**).

The first symptoms in the pathogenicity trials were observed 12-14 days after inoculation on susceptible crop species such as lettuce, lamb's lettuce, spinach, wild rocket and cultivated rocket. Development of symptoms began with chlorosis of leaves and appearance of black or brown streaks in the vascular system, which progressed to plant death at 30-35 days after inoculation. The inoculated strains were reisolated from infected tissues, confirming the Koch's postulates. The re-isolation was performed by using the semi-selective media for Fusaria (Komada 1975) from both symptomless and affected plants (Garibaldi et al. 2004b). Control plants were healthy and did not show any wilting symptoms until the end of the tests (**Fig. 1A-F**). The strains pathogenic on lettuce showed different degree of virulence, with twelve strains showing a disease severity higher than 50% and four strains showing a disease severity between 40 and 50%. The pathogenicity assay showed that, among the races, strains from FOL race 4 were the most virulent, with a disease severity on lettuce cv. Romana Verde ranging from 89% to 95%. All Japanese strains (MAFF 244122, MAFF 744085, and MAFF 744086) from FOL race 3 were highly virulent on lettuce cv. Romana Verde, causing a disease severity ranging from 73 to 95%. Taiwanese strains FOL 18 type 2 (race 1) and FOL 10ATCC (race 2) showed an intermediate pattern with disease severity of 44% and 49%, respectively. Japanese F 9501 strain belonging to FOL race 2 showed 59% disease severity. Italian strains from FOL race 1 inoculated on same lettuce cultivar were more virulent as compared to American strains of FOL race 1. MAFF 244120 of race 1 was slightly virulent and its disease severity was only 17.86%. All strains of FOL races 1 and 2 were only pathogenic on their original lettuce host (**Table 2**).

Additionally, FOL race 3 and FOL race 4 strains were also highly virulent on lamb's lettuce, showing a disease severity ranging from 65.63 to 97.32%.

Characteristic symptoms of Fusarium wilt were observed during the first evaluation at 14 days after inoculation (DAI), with the disease progressively increased over time, reaching the maximum severity at 35 DAI (**Fig. 2** and **Fig. 3**). Among strains MAFF 244122 was the most aggressive strain on lettuce and lamb's lettuce at 14 DAI, 21DaI and 28 DAI. At 35 DAI, FOL 1/16 and Fus. 1.01 induced the highest values of infection on lettuce. While the highest progress of the disease was observed in Fus. Lat. 6/19 and PD 015-04750896 on lamb's lettuce.

Overall, FOL strains of races 3 and 4 caused faster symptom development and plant death, compared to races 1 and 2 (**Fig. 2** and **Fig. 3**). By evaluating the AUDPC, it was observed that the Japanese isolate MAFF 244122 recorded the highest AUDPC, indicating a rapid disease progression in lettuce, while MAFF 244120 (race 1) showed the lowest disease development on lettuce. On lamb's lettuce, isolate MAFF 244122 (race 3) caused fast Fusarium wilt symptom development, whereas the lowest AUDPC was observed in MAFF 744086 as shown in **Table 3**. Only the Italian isolate Fus. Lat. 2/11, originally isolated from lettuce grown in field, was pathogenic on lamb's lettuce, cultivated rocket and wild rocket (**Table 2**), but it was not pathogenic on lettuce, therefore it was assigned to the forma specialis *conglutinans*.

Phylogenetic analysis

Phylogenetic analysis, conducted by sequencing *rpb2* and *tef-1α* genes, on 18 strains of *F. oxysporum* from lettuce and 91 reference strains deposited in GenBank revealed that most strains belonged to the *Fusarium oxysporum* species complex (FOSC), according to Lombard et al. (2019). A further analysis of the selected strains was performed by using concatenated sessions of four loci (*rpb2*, *tef-1α*, *tub2* and *cmdA*). The combined species phylogenetic analysis of FOSC strains consisted

of 109 sequences, including the outgroup sequence from *Fusarium udum* (CBS 177.31) (**Table S2**). A total of 2617 characters (*cmdA*: 1-557, *rpb2*: 564-1440, *tef-1α*: 1447-2066, *tub2*: 2073-2617) were included in phylogenetic analysis. Among the nucleotides, 2217 were constant, 206 were variable and parsimony-uninformative, and 176 were parsimony-informative. A maximum of 1,000 equally MP trees were saved (Tree length = 552, CI = 0.770, RI = 0.886 and RC = 0.682). Bootstrap support values from the MP analysis are included on the Bayesian tree in **Fig. 4**. For the BI, MrModeltest suggested that all partitions should be analyzed with Dirichlet state frequency distributions. The following models were recommended by MrModel test and used: K80for *cmdA*, K80+G for *rpb2*, HKY for *tef-1α* and HKY+G for *tub2*. In the BI, the *cmdA* partition had 34 unique site patterns, the *rpb2* partition had 104 unique site patterns, the *tef-1α* partition had 132 unique site patterns, the *tub2* partition had 158 unique site patterns and the analysis ran for 2,520,000 generations, resulting in 2,521 trees of which 1,891 trees were samples to calculate the posterior probabilities. Multi-locus phylogenetic analysis of 18 strains showed that strains from FOL race 1 and FOL race 4 clustered with *F. curvatum*, together with FOL 10 ATCC of FOL race 2. One isolate of FOL race 2 (F9501) clustered with *F. odoratissimum*. Strains from FOL race 3 and Fus. Lat. 2/11 (*conglutinans* isolate) clustered with *F. cugenangense* and *F. fabacearum*, respectively.

Discussion

The work represents the first assignation of the four races of *F. oxysporum* f. sp. *lactucae* to the species of the *Fusarium oxysporum* species complex (FOSC), according to the nomenclature of Lombard et al. (2019). Strains of *Fusarium oxysporum*, isolated from lettuce in Italy and other countries, were characterized based on pathogenicity on different hosts and on combined phylogenetic analysis of *rpb2*, *tef-1α*, *cmdA* and *tub2*.

Although pathogenicity tests are laborious and time consuming, they are still required because of their effectiveness to differentiate the races in the FOSC (Fujinaga et al. 2001, 2003; Gilardi et al. 2017b). Our results from the pathogenicity test on differential cultivars reveal that Romana Romabella and Costa Rica No. 4 were susceptible to FOL 1/16, which was assigned to race 4, whereas Cencibel and Kiribati were susceptible to Fus. Lat. 4/15 and Fus. Lat. 3/18, which were assigned to race 1. Moreover inoculation experiments on set of differential cultivars showed that Costa Rica No. 4 (resistant to race 1 and susceptible to race 4) and Kiribati (resistant to race 4 and susceptible to race 1) are good cultivars to differentiate the race 1 and race 4 as in previous by Gilardi et al. (2017b), and Tziros and Karaoglanidis (2023). It is interesting to note that strain FOL 1/16 (race 4) was first isolated in Central Italy during the summer of 2016, in a region where lettuce is extensively cultivated, and FOL race 1 was previously the predominant race. While symptoms caused by FOL race 4 were first observed in the full-winter 2018-2019 on severely affected lettuce cvs. Basilio RZ, Flexila, Voltoron, and Descartes. The incidence of the disease ranged from 30 to 100% (Gilardi et al. 2019).

All the tested strains were artificially inoculated on commercially available lettuce cultivars, wild rocket, cultivated rocket, lamb's lettuce, and spinach to confirm if they are pathogenic only on their original host or also on other leafy vegetables. Moreover, pathogenic strains of *Fusarium oxysporum* are differentiated into *formae speciales* based on their host range, which is normally restricted to only one or a few plant species. Using this approach, four races of FOL were tested on members of the plant families Asteraceae, Brassicaceae and Amaranthaceae using a cross-pathogenicity test. The pathogenicity test revealed that 17 out of 18 *Fusarium oxysporum* strains developed wilt symptoms on lettuce. All strains of race 3 and race 4 out of the 17 strains pathogenic on lettuce were also pathogenic on lamb’s lettuce with different levels of disease severity. MAFF 244122 strain was more virulent on lamb’s lettuce, whereas Fus. Lat. 3/18 strain was more virulent on lettuce. Previous

studies proved that the agents of Fusarium wilt could be pathogenic on other salad crops besides lettuce, e.g. Fusarium wilt was regularly isolated from lamb's lettuce, cultivated and wild rocket, in the same area where FOL was isolated (Catti et al. 2007; Garibaldi et al. 2004b, 2006; Gilardi et al. 2008; Gullino et al. 2004). Overall, strains of races 3 and 4 were statistically more virulent on lettuce compared to strains of races 1 and 2. After inoculation, FOL infects the roots and enters the xylem vessels, and is ultimately translocated to the shoots by water movement (Scott et al. 2014). The xylem becomes obstructed, resulting in plant yellowing and wilting (Subbarao et al. 2017). Fusarium wilt symptoms are influenced by several factors such as soil temperature, plant age, planting date, genetic background of the plant, inoculum and virulence of the pathogen strains (Gilardi et al. 2021; Matheron et al. 2005; Scott et al. 2010; Paugh and Gordon 2019; Spadaro and Gullino 2022).

One isolate, Fus. Lat. 2/11, originally isolated from lettuce, showed to be not pathogenic on lettuce, but was pathogenic on wild and cultivated rocket, and lamb's lettuce, and was therefore assigned to the *forma specialis conglutinans*. *F. oxysporum* f. sp. *conglutinans* and *raphani* previously showed an expanded host range not limited only to the Brassicaceae family, but also lamb's lettuce (Srinivasan et al. 2010; 2012). The isolation of *F. oxysporum* f. sp. *conglutinans* from lettuce could be favored by the internalization process of strains of *F. oxysporum* in lettuce: the endophytic stage is unable to cause disease symptoms, but the colonization of non-host plants can contribute to the increase of the inoculum in the soil, particularly in fields that are rotated with other crops. This was suggested by Scott et al. (2014), who documented root colonization of broccoli, cauliflower, and spinach by FOL. The frequency of infection was significantly lower on the three rotation crops, than on a susceptible lettuce cultivar without rotation. Another possible reason is that lettuce, lamb's lettuce, wild rocket, and cultivated rocket are all grown intensively in the same region and share the same soil conditions. The capability of the *forma specialis* conglutinans to target lettuce could have only become apparent once

varieties of this species that are highly susceptible were introduced and grown in intensive agricultural practices.

A multilocus sequence analysis approach was used for the correct identification and distinction of species boundaries in the *Fusarium* genus (Edwards et al. 2016; O'Donnell et al. 2010). Most of the previous studies showed that *Fusarium* strains were identified based on morphology and *tef-1α* gene sequence (Claerbout et al. 2018; Garibaldi et al. 2002; Gilardi et al. 2017a; Guerrero et al. 2020; Pasquali et al. 2007). Lombard et al. (2019) recommended that at least *rpb2* and *tef-1α* regions should be used for identification of *Fusarium* at species level. In the multilocus phylogenetic analysis, based on *rpb2*, *tef-1α*, *tub2* and *cmdA* regions, strains of FOL race 1 from France, Italy, Japan, Taiwan and the USA grouped in the same clade with strains of race 4 (Belgium, Italy and the Netherlands) and a Taiwanese isolate from race 2. The studies of Claerbout et al. (2018; 2023), Gilardi et al. (2017b), and Tziros and Karaoglanidis (2023) already reported that strains from race 1 and race 4 belong to the same clade. In our study, Japanese strains from race 3 formed a separate clade in agreement with Fujinaga et al. (2005). One Japanese isolate from race 2 clustered in a separate clade from race 1, confirming that races 1 and 2 belong to two different genetic linages (Mbofung et al. 2007). Previously, Fujinaga et al. (2005) revealed that races 1, 2 and 3 are genetically different, and they belong to different clades. In our phylogenetic analysis, Taiwanese and Japanese strains from race 2 belonged to separate clades, possibly due to the different geographical origin (Fujinaga et al. 2005).

This study confirmed that there was significant genetic variation between strains of the four races of FOL. The strains of FOL were assigned for the first time to the species *F. curvatum*, *F. cugenangense*, and *F. odoratissimum*. The species *F. odoratissimum* includes not only the isolate F9501 of FOL race 2, but also the causal agents known as f. sp. *cubense* which cause wilting in *Musa* sp. While the *F. curvatum* hosts strains of race 1 and 4, and the pathogens f. sp. *matthiolae* and f. sp.

meniscoideum which lead to wilting in *Matthiola incana* and *Beaucarnia* sp., respectively. Our results showed that *Fusarium* strains pathogenic on lettuce belonged to three distinct species (Lombard et al. 2019), with different host range and level of virulence.

The new species nomenclature was unable to separate the four races of FOL, and even the *formae speciales* could not be clearly separated. The strains of race 2 are divided into two species, i.e. *F. odoratissimum* and *F. curvatum*, whereas the strains of races 1 and 4 (and one isolate of race 2) are in the same species, i.e. *F. curvatum*. This study demonstrates that the phylogenetic analysis permits the separation of some races of FOL, but alone it is insufficient, therefore it should be accompanied by biological assays based on pathogenicity. Current study enhances our understanding of the *Fusarium oxysporum* species complex (FOSC) by classifying four races of *F. oxysporum* f. sp. *lactucae* and revealing their distinct genetic profiles and host range preferences. The identification of specific lettuce and lamb's lettuce cultivars susceptible to certain FOL races provides actionable insights for agricultural producers. An integrated disease management strategies such as crop rotation, healthy seed and propagation material along with resistant cultivars should be used to control the Fusarium wilt of lettuce.

Funding

This work was partially funded by the project "A gnotobiotic-based approach to unravel the role of the plant microbiome and develop synthetic communities increasing plant growth and stress tolerance - NATURE" (PRIN 2017 Program).

Acknowledgements

This work was partially funded by the project "A gnotobiotic-based approach to unravel the role of the plant microbiome and develop synthetic communities increasing plant growth and stress tolerance - NATURE" (PRIN 2017 Program).

The Authors would like to thank Dr M. H. Fujinaga (Nagano Vegetable and Ornamental Crops Experiment Station, Nagano, Japan) for sharing the Japanese isolates of the pathogen, Johan Meffert for sharing the Dutch Fol isolates, and Prof. M. Höfte, Department of Plants and Crops, Laboratory of Phytopathology, Ghent University, Belgium for sharing the Belgian isolate.

Table 1: Information of *Fusarium oxysporum* isolates from lettuce used in this study: *forma specialis*, race, year of isolation, origin, and references.

Isolate names	*F. oxysporum* f. sp. and Race[1]	Year of Isolation	Origin	References[2]
Fus. Lat. 4/15	*F. oxysporum* f. sp. *lactucae* R1	2015	Italy	This study
Fus. Lat. 3/18	*F. oxysporum* f. sp. *lactucae* R1	2018	Italy	This study
Fus. Lat. 7/18	*F. oxysporum* f. sp. *lactucae* R1	2018	Italy	Gilardi et al., 2021
MAFF 244120	*F. oxysporum* f. sp. *lactucae* R1	1996	Japan	Pasquali et al. 2007
JCP 0043	*F. oxysporum* f. sp. *lactucae* R1	2002	USA	Pasquali et al. 2007
AZ-01	*F. oxysporum* f. sp. *lactucae* R1	2002	USA	Pasquali et al. 2007
FOL 8/16	*F. oxysporum* f. sp. *lactucae* R1	2016	France	Gilardi et al. 2017
FOL 18-Type 2	*F. oxysporum* f. sp. *lactucae* R1	2005	Taiwan	Pasquali et al. 2007
F 9501	*F. oxysporum* f. sp. *lactucae* R2	1995	Japan	Pasquali et al. 2007
FOL 10 ATCC	*F. oxysporum* f. sp. *lactucae* R2	unknown	Taiwan	Pasquali et al. 2007
MAFF 744085	*F. oxysporum* f. sp. *lactucae* R3	2002	Japan	Gilardi et al, 2017
MAFF 744086	*F. oxysporum* f. sp. *lactucae* R3	2002	Japan	Gilardi et al. 2017
MAFF 244122	*F. oxysporum* f. sp. *lactucae* R3	2010	Japan	Gilardi et al. 2017
FOL 1/16	*F. oxysporum* f. sp. *lactucae* R4	2016	Italy	This study
Fus. Lat. 6/19	*F. oxysporum* f. sp. *lactucae* R4	2019	Italy	Gilardi et al. 2019
Fus. 1.01	*F. oxysporum* f. sp. *lactucae* R4	2018	Belgium	Claerbout et al. 2018
PD 015-04750896	*F. oxysporum* f. sp. *lactucae* R4	2015	Netherlands	Gilardi et al. 2017
Fus. Lat. 2/11	*F. oxysporum* f. sp. *conglutinans*	2011	Italy	This study

[1] R1: race 1; R2: race 2; R3: race 3; R4: race 4, [2] data of pathogenicity for race determination.

Table 2. Data of pathogenicity of *Fusarium oxysporum* isolates on lettuce, lamb's lettuce, cultivated rocket, wild rocket and spinach used in this study: race, disease severity, standard deviation, and standard error.

Isolates	Race	Lettuce*			Lamb's Lettuce*			Cultivated Rocket*	Wild Rocket*	Spinach*
		DS%[1]	SD[2]	SE[3]	DS%	SD	SE	DS%	DS%	DS%
Fus. Lat. 04/15	R1	54.91$^{c\text{-}f}$**	5.13	2.56	0.00^{a}**	0	0	0	0	0
Fus. Lat. 3/18	R1	70.54$^{d\text{-}g}$	6.6	3.3	0.00^{a}	0	0	0	0	0
Fus. Lat. 7/18	R1	62.50$^{c\text{-}f}$	20.41	10.21	0.00^{a}	0	0	0	0	0
MAFF 244120	R1	17.86^{ab}	5.26	2.63	0.00^{a}	0	0	0	0	0
JCP 043	R1	40.18^{bc}	10.76	5.38	0.00^{a}	0	0	0	0	0
AZ-01	R1	47.32^{cd}	10.36	5.18	0.00^{a}	0	0	0	0	0
FOL 8/16	R1	60.27$^{c\text{-}f}$	13.39	6.7	0.00^{a}	0	0	0	0	0
FOL 18-Type 2	R1	44.64 c	6.19	3.09	0.00^{a}	0	0	0	0	0
F 9501	R2	59.38$^{c\text{-}f}$	15.04	7.52	0.00^{a}	0	0	0	0	0
FOL 10 ATCC	R2	49.11$^{c\text{-}e}$	11.71	5.85	0.00^{a}	0	0	0	0	0
MAFF 744085	R3	77.68^{fg}	12.07	6.03	74.11^{bc}	9.39	4.7	0	0	0
MAFF 744086	R3	73.21$^{e\text{-}g}$	11.57	5.79	65.63^{b}	14.31	7.16	0	0	0
MAFF 244122	R3	95.09^{g}	2.25	1.12	97.32^{e}	3.42	1.71	0	0	0

FOL 1/16	R4	95.54^{g}	2.31	1.15	90.63 ^{de}	7.2	3.6	0	0	0
Fus. Lat. 6/19	R4	93.75^{g}	7.22	3.61	94.20 ^{de}	7.2	3.6	0	0	0
Fus. 1.01	R4	95.54^{g}	4.25	2.13	82.59 ^{cd}	10.55	5.28	0	0	0
PD 015-04750896	R4	89.73^{g}	6.74	3.37	94.64 ^{de}	6.36	3.18	0	0	0
Fus. Lat. 02/11		0.00^{a}	0	0	87.50 ^{cd}	6.52	3.26	84.82	24.11	0

[1] DS%: Disease severity in percentage; [2] SD.: standard deviation; [3] SE: standard error; R1: race 1; R2: race 2; R3: race 3; R4: race 4 *Data are the average of three trials. **Data of the same column with the same letter are not significantly different according to Tukey's test ($P<0.05$).

Table 3: Area under disease progress curve (AUDPC) of *Fusarium oxysporum* isolates on lettuce and lamb's lettuce.

Isolates	AUDPC[1]					
	Lettuce*	SD[2]	SE[3]	Lamb's Lettuce*	SD	SEM
Fus. Lat. 04/15	678.13$^{b\text{-}f**}$	193.41	96.71	0.00^{a**}	0.00	0.00
Fus. Lat. 3/18	1187.50^{fg}	188.81	94.41	0.00^{a}	0.00	0.00
Fus. Lat. 7/18	1070.31$^{e\text{-}g}$	487.83	243.92	0.00^{a}	0.00	0.00
MAFF 244120	240.63^{ab}	87.57	43.79	0.00^{a}	0.00	0.00
JCP 043	546.88$^{b\text{-}d}$	246.04	123.02	0.00^{a}	0.00	0.00
AZ-01	517.19$^{b\text{-}d}$	121.88	60.94	0.00^{a}	0.00	0.00
FOL 8/16	1032.81$^{d\text{-}g}$	293.03	146.52	0.00^{a}	0.00	0.00
FOL 18-Type 2	367.19^{ab}	15.63	7.81	0.00^{a}	0.00	0.00
F 9501	950.00$^{c\text{-}g}$	264.43	132.21	0.00^{a}	0.00	0.00
FOL 10 ATCC	573.44$^{b\text{-}e}$	200.41	100.21	0.00^{a}	0.00	0.00
MAFF 744085	1212.50^{g}	256.43	128.21	425.00^{bc}	156.71	78.35
MAFF 744086	1293.75^{g}	245.53	122.77	291.00^{b}	90.75	45.38
MAFF 244122	2253.13^{h}	136.98	68.49	793.75^{d}	567.73	283.87
FOL 1/16	2125.00^{h}	298.83	149.41	630.79$^{b\text{-}d}$	510.28	255.14

Fus. Lat. 6/19	1825.00[h]	190.05	95.03	470.31[b-d]	329.83	164.91
Fus. 1.01	1181.25[fg]	106.80	53.40	556.25[b-d]	371.41	185.70
PD 015-04750896	1085.94[e-g]	275.63	137.82	689.06[cd]	303.77	151.88
Fus. Lat. 02/11	0.00[a]	0.00	0.00	629.69[cd]	389.36	194.68
Control	0.00[a]	0.00	0.00	0.00[a]	0.00	0.00

[1] AUDPC: area under disease progress curve. [2] SD.: standard deviation; [3] SE: standard error. *Data are the average of three trials. **Data of the same column with the same letter are not significantly different according to Tukey's test ($P<0.05$).

Figures

Fig. 1. Pathogenicity trial of FOL isolates on lettuce (cv. Romana Verde, ISI Sementi), lamb's lettuce (cv. Trophy, RB Sementi), spinach (cv. Zebu, Rijk Zwaan), wild rocket (cv. Winter, Orosem), cultivated rocket (cv. Coltivata, Furia Sementi). **A-B)** Dead plants of lettuce and lamb's lettuce **C)** wilt symptoms on lettuce **D)** healthy plants **E)** dead plants of lettuce **F)** wilt symptoms on lamb's lettuce.

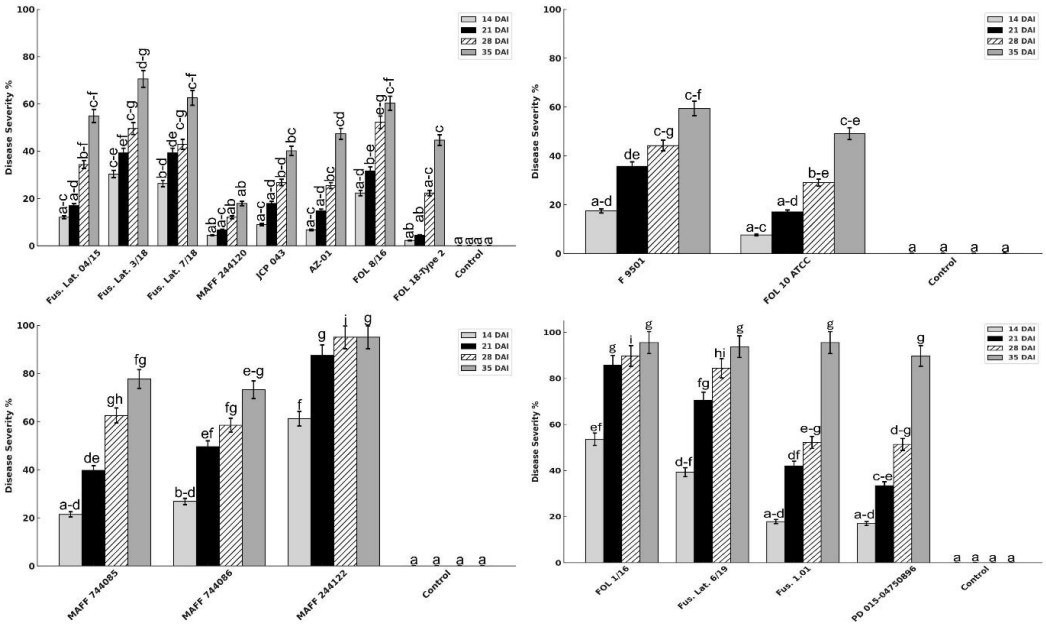


Fig. 2. Disease development progress for disease severity of *Fusarium oxysporum* f. sp. *lactucae* on lettuce. DAI: days after inoculation. Bars with the same letter above are not significantly different according to Tukey's test ($P<0.05$).

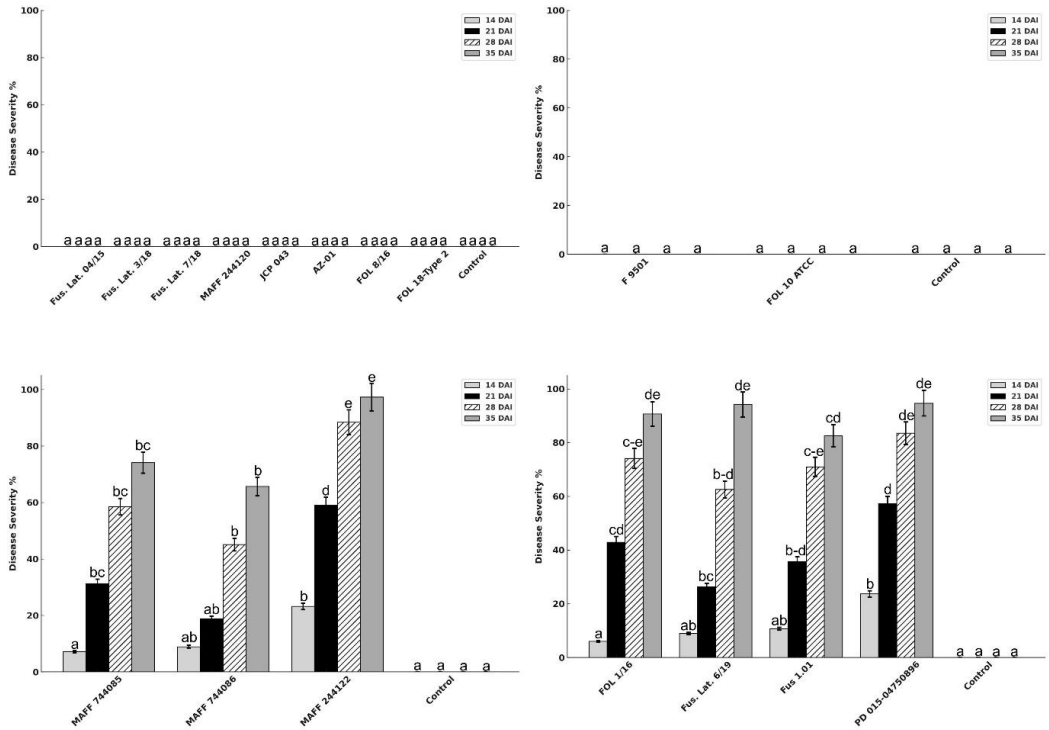


Fig. 3. Disease development progress for disease severity of *Fusarium oxysporum* f. sp. *lactucae* on lamb's lettuce. DAI: days after inoculation. Bars with the same letter above are not significantly different according to Tukey's test ($P<0.05$).

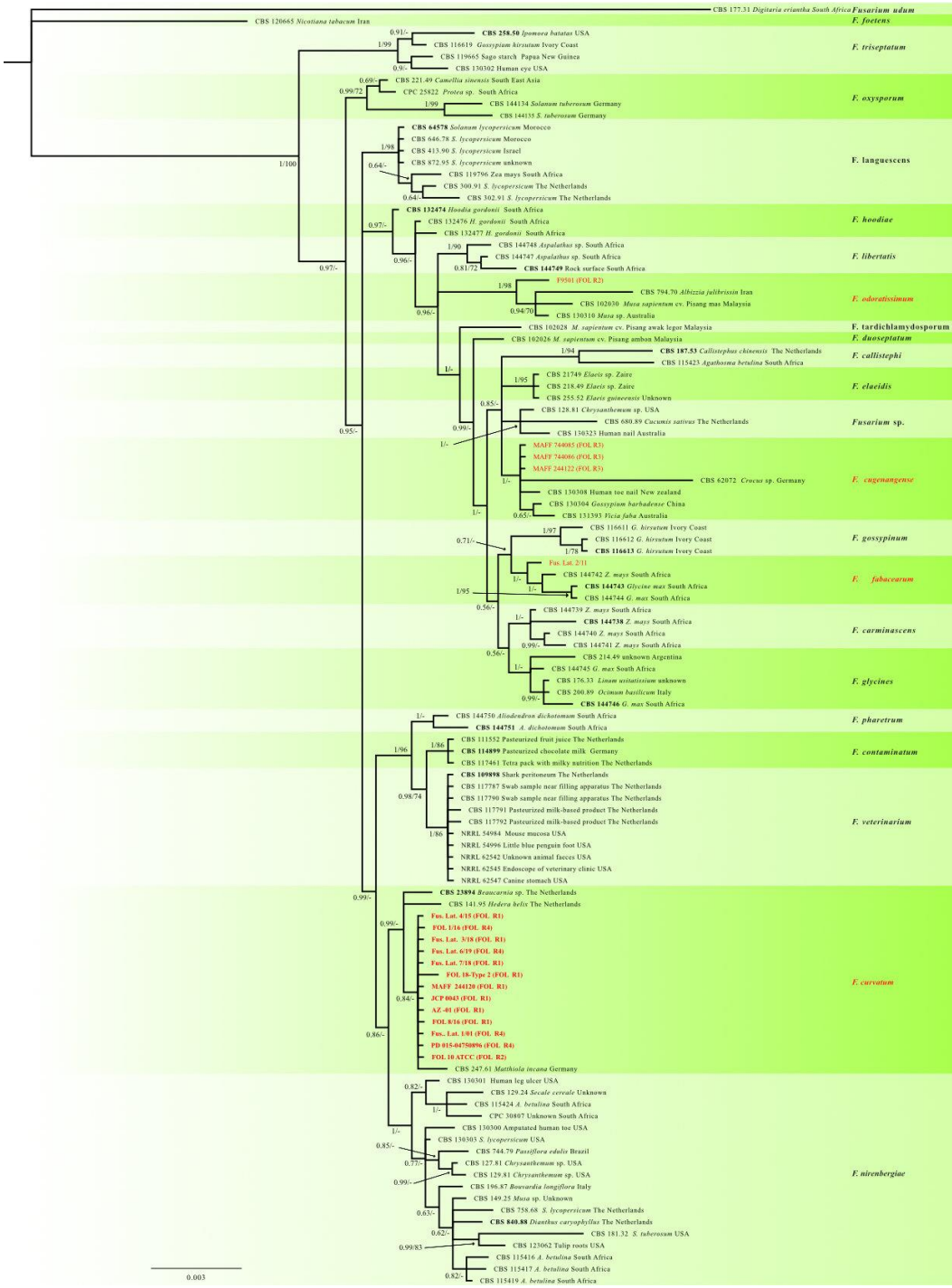


Fig. 4. Consensus phylogram of 1,891 trees resulting from a Bayesian analysis of the combined *rpb2, tef-1α, cmdA* and *tub2* sequence alignments of the *Fusarium*

species. Bootstrap support values and Bayesian posterior probability values are indicated at the nodes. Host and country of origin are listed next to the strain numbers. Ex-type isolates are indicated in bold. The isolates obtained in this study are in red. The tree was rooted with *Fusarium udum* (CBS 177.31).

e-Xtra (Supplementary) Files

Table S1: Pathogenicity of *Fusarium oxysporum* f. sp. *lactucae* on differential lettuce cultivars to determine the physiological races.

Isolate names	Race	Pathogenicity on differential cultivars[1]							
		Romana Romabella[2]		Costa Rica No. 4[3]		Cencibel[4]		Kiribati[5]	
		DI%	DS%	DI%	DS%	DI%	DS%	DI%	DS%
Fus. Lat. 4/15	1	0^{a}*	0a	0^{a}	0^{a}	67^{b}	33^{b}	100^{b}	92^{b}
Fus. Lat. 3/18	1	0^{a}	0a	0^{a}	0^{a}	67^{b}	38^{b}	100^{b}	96^{b}
FOL 1/16	4	83^{b}	29^{b}	100^{b}	92^{b}	0^{a}	0^{a}	0^{a}	0^{a}
Fus. Lat. 6/19	4	83^{b}	25^{b}	100^{b}	58^{b}	0^{a}	0^{a}	0^{a}	0^{a}
Fus. 1.01	4	100^{b}	50^{b}	100^{b}	79^{b}	0^{a}	0^{a}	0^{a}	0^{a}
Healthy		0^{a}	0^{a}	0^{a}	0.0^{a}	0^{a}	0^{a}	0^{a}	0^{a}

[1] race classification system (Fujinaga et al. 2001, 2003); [2] Romana Romabella: susceptible to race 4 and resistant to race 1; [3] Costa Rica No. 4: susceptible to race 4 and resistant to race 1; [4] Cencibel: susceptible to race 1 and resistant to race 4; [5] Kiribati: susceptible to race 1 and resistant to race 4. *Data are the average of two trials. Data of the same column with the same letter are not significantly different according to Tukey's test ($P<0.05$).

Table S2. List of 109 representative isolates of *Fusarium* spp. including the outgroup *Fusarium udum* used in phylogenetic analyses for this study, with details about species, strains, origin, special form, and GenBank accession numbers.

					GenBank accession			
Species	**Strain[1]**	**Host**	**Special form**	**Origin**	**RPB2**	**TEF**	**cmdA**	**tub2**
Fusarium callistephi	CBS 187.53^{T}	*Callistephus chinensis*	*callistephi*	The Netherlands	MH484875	MH484966	MH484693	MH485057
F. callistephi	CBS 115423	*Agathosma betulina*	-	South Africa	MH484905	MH484996	MH484723	MH485087
F. carminascens	CBS 144739 = CPC 25792	*Zea mays*	-	South Africa	MH484934	MH485025	MH484752	MH485116
F. carminascens	CBS 144740 = CPC 25793	*Z. mays*	-	South Africa	MH484935	MH485026	MH484753	MH485117
F. carminascens	CBS 144741 = CPC 25795	*Z. mays*	-	South Africa	MH484936	MH485027	MH484754	MH485118
F. carminascens	CBS 144738 = CPC 25800^{T}	*Z. mays*	-	South Africa	MH484937	MH485028	MH484755	MH485119
F. contaminatum	CBS 111552	Pasteurized fruit juice	-	The Netherlands	MH484900	MH484991	MH484718	MH485082
F. contaminatum	CBS 114899^{T}	Pasteurized chocolate milk	-	Germany	MH484901	MH484992	MH484719	MH485083
F. contaminatum	CBS 117461	Tetra pack with milky nutrition	-	The Netherlands	MH484911	MH485002	MH484729	MH485093
F. cugenangense	CBS 620.72 = DSM 11271 = NRRL 36520	*Crocus* sp.	*gladioli*	Germany	MH484879	MH484970	MH484697	MH485061
F. cugenangense	CBS 130304 = BBA 69050 = NRRL 25433	*Gossypium barbadense*	*vasinfectum*	China	MH484921	MH485012	MH484739	MH485103
F. cugenangense	CBS 130308 = ATCC 26225 = NRRL 25387	Human toenail	-	New Zealand	MH484920	MH485011	MH484738	MH485102
F. cugenangense	CBS 131393	*Vicia faba*	-	Australia	MH484928	MH485019	MH484746	MH485110

F. cugenangense	MAFF 744085	*Lactuca sativa*	*lactucae*	Japan	*OP918966*	*OP918950*	*OP947637*	*OP899654*
F. cugenangense	MAFF 744086	*L. sativa*	*lactucae*	Japan	*OP918967*	*OP918951*	*OP947638*	*OP899655*
F. cugenangense	MAFF 244122	*L. sativa*	*lactucae*	Japan	*OP918968*	-	*OP947639*	*OP899656*
F. curvatum	CBS 247.61 = BBA 8398 = DSM 62308 = NRRL 22545	*Matthiola incana*	*matthiolae*	Germany	MH484876	MH484967	MH484694	MH485058
F. curvatum	CBS 238.94 = NRRL 26422 = PD 94/184^{T}	*Beaucarnia* sp.	*meniscoideum*	The Netherlands	MH484893	MH484984	MH484711	MH485075
F. curvatum	CBS 141.95 = NRRL 36251 = PD 94/1518	*Hedera helix*	-	The Netherlands	MH484894	MH484985	MH484712	MH485076
F. curvatum	Fus. Lat. 04/15	*L. sativa*	*lactucae*	Italy	*OP918956*	*OP918941*	*OP947627*	*OP899644*
F. curvatum	Fus. Lat. 3/18	*L. sativa*	*lactucae*	Italy	*OP918957*	*OP918942*	*OP947628*	*OP899645*
F. curvatum	Fus. Lat. 7/18	*L. sativa*	*lactucae*	Italy	*OP918958*	*OP918943*	*OP947629*	*OP899646*
F. curvatum	MAFF 244120	*L. sativa*	*lactucae*	Japan	*OP918959*	*OP918944*	*OP947630*	*OP899647*
F. curvatum	JCP 0043	*L. sativa*	*lactucae*	USA	*OP918960*	*OP918945*	*OP947631*	*OP899648*
F. curvatum	AZ-01	*L. sativa*	*lactucae*	USA	*OP918961*	*OP918946*	*OP947632*	*OP899649*
F. curvatum	FOL 8/16	*L. sativa*	*lactucae*	France	*OP918962*	KY009875	*OP947633*	*OP899650*
F. curvatum	FOL 18-Type 2	*L. sativa*	*lactucae*	Taiwan	*OP918963*	*OP918947*	*OP947634*	*OP899651*
F. curvatum	FOL 10 ATCC	*L. sativa*	*lactucae*	Taiwan	*OP918965*	*OP918949*	*OP947636*	*OP899653*
F. curvatum	FOL 1/16	*L. sativa*	*lactucae*	Italy	*OP918969*	*OP918952*	*OP947640*	-

F. curvatum	Fus. Lat. 6/19	*L. sativa*	*lactucae*	Italy	*OP918970*	*OP918953*	*OP947641*	*OP899657*
F. curvatum	Fus 1.01	*L. sativa*	*lactucae*	Belgium	*OP918971*	*OP918954*	*OP947642*	*OP899658*
F. curvatum	PD 015-04750896	*L. sativa*	*lactucae*	Netherland	*OP918972*	KU840923	*OP947643*	*OP899659*
F. duoseptatum	CBS 102026 = NRRL 36115	*Musa sapientum* cv. *Pisang ambon*	*cubense*	Malaysia	MH484896	MH484987	MH484714	MH485078
F. elaeidis	CBS 217.49 = NRRL 36358	*Elaeis sp.*	*elaeidis*	Zaire	MH484870	MH484961	MH484688	MH485052
F. elaeidis	CBS 218.49 = NRRL 36359	*Elaeis sp.*	*elaeidis*	Zaire	MH484871	MH484962	MH484689	MH485053
F. elaeidis	CBS 255.52 = NRRL 36386	*Elaeis guineensis*	*elaeidis*	Unknown	MH484874	MH484965	MH484692	MH485056
F. fabacearum	CBS 144742 = CPC 25801	*Z. mays*	-	South Africa	MH484938	MH485029	MH484756	MH485120
F. fabacearum	CBS 144743 = CPC 25802^{T}	*Glycine max*	-	South Africa	MH484939	MH485030	MH484757	MH485121
F. fabacearum	CBS 144744 = CPC 25803	*G. max*	-	South Africa	MH484940	MH485031	MH484758	MH485122
F. fabacearum	Fus. Lat. 2/11	*L. sativa*	*lactucae*	Italy	*OP918973*	*OP918955*	*OP947644*	*OP947645*
F. foetens	CBS 120665	*Nicotiana tabacum*	-	Iran	MH484918	MH485009	MH484736	MH485100
F. glycines	CBS 176.33 = NRRL 36286	*Linum usitatissium*	*lini*	Unknown	MH484868	MH484959	MH484686	MH485050
F. glycines	CBS 214.49 = NRRL 36356	Unknown	-	Argentina	MH484869	MH484960	MH484687	MH485051
F. glycines	CBS 200.89	*Ocimum basilicum*	*basilici*	Italy	MH484888	MH484979	MH484706	MH485070
F. glycines	CBS 144745 = CPC 25804	*G. max*	-	South Africa	MH484941	MH485032	MH484759	MH485123
F. glycines	CBS 144746 = CPC 25808^{T}	*G. max*	-	South Africa	MH484942	MH485033	MH484760	MH485124
F. gossypinum	CBS 116611	*Gossypium hirsutum*	*vasinfectum*	Ivory Coast	MH484907	MH484998	MH484725	MH485089

F. gossypinum	CBS 116612	*G. hirsutum*	*vasinfectum*	Ivory Coast	MH484908	MH484999	MH484726	MH485090
F. gossypinum	CBS 116613^{T}	*G. hirsutum*	*vasinfectum*	Ivory Coast	MH484909	MH485000	MH484727	MH485091
F. hoodiae	CBS 132474^{T}	*Hoodia gordonii*	*hoodiae*	South Africa	MH484929	MH485020	MH484747	MH485111
F. hoodiae	CBS 132476	*H. gordonii*	*hoodiae*	South Africa	MH484930	MH485021	MH484748	MH485112
F. hoodiae	CBS 132477	*H. gordonii*	*hoodiae*	South Africa	MH484931	MH485022	MH484749	MH485113
F. languescens	CBS 645.78 = NRRL 36531^{T}	*Solanum lycopersicum*	*lycopersici*	Morocco	MH484880	MH484971	MH484698	MH485062
F. languescens	CBS 646.78 = NRRL 36532	*S. lycopersicum*	*lycopersici*	Morocco	MH484881	MH484972	MH484699	MH485063
F. languescens	CBS 413.90 = ATCC 66046 = NRRL 36465	*S. lycopersicum*	*lycopersici*	Israel	MH484890	MH484981	MH484708	MH485072
F. languescens	CBS 300.91 = NRRL 36416	*S. lycopersicum*	*lycopersici*	The Netherlands	MH484891	MH484982	MH484709	MH485073
F. languescens	CBS 302.91 = NRRL 36419	*S. lycopersicum*	*lycopersici*	The Netherlands	MH484892	MH484983	MH484710	MH485074
F. languescens	CBS 872.95 = NRRL 36570	*S. lycopersicum*	*radicis-lycopersici*	Unknown	MH484895	MH484986	MH484713	MH485077
F. languescens	CBS 119796 = MRC 8437	*Z. mays*	-	South Africa	MH484917	MH485008	MH484735	MH485099
F. libertatis	CBS 144748 = CPC 25782	*Aspalathus* sp.	-	South Africa	MH484932	MH485023	MH484750	MH485114
F. libertatis	CBS 144747 = CPC 25788	*Aspalathus* sp.	-	South Africa	MH484933	MH485024	MH484751	MH485115
F. libertatis	CBS 144749 = CPC 28465^{T}	*Rock surface*	-	Africa	MH484944	MH485035	MH484762	MH485126
F. nirenbergiae	CBS 129.24	*Secale cereale*	-	Unknown	MH484864	MH484955	MH484682	MH485046
F. nirenbergiae	CBS 149.25 = NRRL 36261	*Musa* sp.	*cubense*	Unknown	MH484865	MH484956	MH484683	MH485047

F. nirenbergiae	CBS 181.32 = NRRL 36303	*S. tuberosum*	-	USA	MH484867	MH484958	MH484685	MH485049
F. nirenbergiae	CBS 758.68 = NRRL 36546	*S. lycopersicum*	*lycopersici*	The Netherlands	MH484877	MH484968	MH484695	MH485059
F. nirenbergiae	CBS 744.79 = BBA 62355 = NRRL 22549	*Passiflora edulis*	*passiflorae*	Brazil	MH484882	MH484973	MH484700	MH485064
F. nirenbergiae	CBS 127.81 = BBA 63924 = NRRL 36229	*Chrysanthemum sp.*	*chrysanthemi*	USA	MH484883	MH484974	MH484701	MH485065
F. nirenbergiae	CBS 129.81 = BBA 63926 = NRRL 22539	*Chrysanthemum* sp.	*chrysanthemi*	USA	MH484885	MH484976	MH484703	MH485067
F. nirenbergiae	CBS 196.87 = NRRL 26219	*Bouvardia longiflora*	*bouvardiae*	Italy	MH484886	MH484977	MH484704	MH485068
F. nirenbergiae	CBS 840.88^{T}	*Dianthus caryophyllus*	*dianthi*	The Netherlands	MH484887	MH484978	MH484705	MH485069
F. nirenbergiae	CBS 115416 = CPC 5307	*Agathosma betulina*	-	South Africa	MH484902	MH484993	MH484720	MH485084
F. nirenbergiae	CBS 115417 = CPC 5306	*A. betulina*	-	South Africa	MH484903	MH484994	MH484721	MH485085
F. nirenbergiae	CBS 115419 = CPC 5308	*A. betulina*	-	South Africa	MH484904	MH484995	MH484722	MH485086
F. nirenbergiae	CBS 115424 = CPC 5312	*A. betulina*	-	South Africa	MH484906	MH484997	MH484724	MH485088
F. nirenbergiae	CBS 123062 = GJS 91-17	Tulip roots	-	USA	MH484919	MH485010	MH484737	MH485101
F. nirenbergiae	CBS 130300 = NRRL 26368	Amputated human toe	-	USA	MH484925	MH485016	MH484743	MH485107
F. nirenbergiae	CBS 130301 = NRRL 26374	Human leg ulcer	-	USA	MH484926	MH485017	MH484744	MH485108
F. nirenbergiae	CBS 130303	*S. lycopersicum*	*radicis-lycopersici*	USA	MH484923	MH485014	MH484741	MH485105
F. nirenbergiae	CPC 30807	unknown	-	South Africa	MH484950	MH485041	MH484768	MH485132

F. odoratissimum	CBS 794.70 = BBA 11103 = NRRL 22550	*Albizzia julibrissin*	*perniciosum*	Iran	MH484878	MH484969	MH484696	MH485060
F. odoratissimum	CBS 102030	*M. sapientum cv. Pisang mas*	*cubense*	Malaysia	MH484898	MH484989	MH484716	MH485080
F. odoratissimum	CBS 130310 = NRRL 25603	*Musa* sp.	*cubense*	Australia	MH484922	MH485013	MH484740	MH485104
F. odoratissimum	F 9501	*L. sativa*	*lactucae*	Japan	OP918964	OP918948	OP947635	OP899652
F. oxysporum	CBS 221.49 = IHEM 4508 = NRRL 22546	*Camellia sinensis*	*medicaginis*	South East Asia	MH484872	MH484963	MH484690	MH485054
F. oxysporum	CBS 144134^{T}	*S. tuberosum*	-	Germany	MH484953	MH485044	MH484771	MH485135
F. oxysporum	CBS 144135	*S. tuberosum*	-	Germany	MH484954	MH485045	MH484772	MH485136
F. oxysporum	CPC 25822	*Protea* sp.	-	South Africa	MH484943	MH485034	MH484761	MH485125
F. pharetrum	CBS 144750 = CPC 30822	*Aliodendron dichotomum*	-	South Africa	MH484951	MH485042	MH484769	MH485133
F. pharetrum	CBS 144751 = CPC 30824^{T}	*A. dichotomum*	-	South Africa	MH484952	MH485043	MH484770	MH485134
F. trachichlamydosporum	CBS 102028 = NRRL 36117	*M. sapientum* cv. *Pisang awak legor*	*cubense*	Malaysia	MH484897	MH484988	MH484715	MH485079
F. triseptatum	CBS 258.50 = NRRL 36389^{T}	*Ipomoea batatas*	*batatas*	USA	MH484873	MH484964	MH484691	MH485055
F. triseptatum	CBS 116619	*G. hirsutum*	*vasinfectum*	Ivory Coast	MH484910	MH485001	MH484728	MH485092
F. triseptatum	CBS 119665	Sago starch	-	Papua New Guinea	MH484916	MH485007	MH484734	MH485098
F. triseptatum	CBS 130302 = NRRL 26360 = FRC 755	Human eye	-	USA	MH484924	MH485015	MH484742	MH485106
F. udum	CBS 177.31	*Digitaria eriantha*	-	South Africa	MH484866	MH484957	MH484684	MH485048
F. veterinarium	CBS 109898 = NRRL 36153^{T}	Shark peritoneum	-	The Netherlands	MH484899	MH484990	MH484717	MH485081

F. veterinarium	CBS 117787	Swab sample near filling apparatus	-	The Netherlands	MH484912	MH485003	MH484730	MH485094
F. veterinarium	CBS 117790	Swab sample near filling apparatus	-	The Netherlands	MH484913	MH485004	MH484731	MH485095
F. veterinarium	CBS 117791	Pasteurized milk-based product	-	The Netherlands	MH484914	MH485005	MH484732	MH485096
F. veterinarium	CBS 117792	Pasteurized milk-based product	-	The Netherlands	MH484915	MH485006	MH484733	MH485097
F. veterinarium	NRRL 54984	*Mouse mucosa*	-	USA	MH484945	MH485036	MH484763	MH485127
F. veterinarium	NRRL 54996	Little blue penguin foot	-	USA	MH484946	MH485037	MH484764	MH485128
F. veterinarium	NRRL 62542	Unknown animal faeces	-	USA	MH484947	MH485038	MH484765	MH485129
F. veterinarium	NRRL 62545	Endoscope of veterinary clinic	-	USA	MH484948	MH485039	MH484766	MH485130
F. veterinarium	NRRL 62547	Canine stomach	-	USA	MH484949	MH485040	MH484767	MH485131
Fusarium sp.	CBS 128.81 = BBA 63925 = NRRL 36233	*Chrysanthemum* sp.	*chrysanthemi*	USA	MH484884	MH484975	MH484702	MH485066
Fusarium sp.	CBS 680.89 = NRRL 26221	*Cucumis sativus*	*cucurbitacearum*	The Netherlands	MH484889	MH484980	MH484707	MH485071
Fusarium sp.	CBS 130323	Human nail	-	Australia	MH484927	MH485018	MH484745	MH485109

1 ATCC: American Type Culture Collection, USA; BBA: Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin-Dahlem, Germany; CBS: Westerdijk Fungal Biodiverity Institute (WIFB), Utrecht, The Netherlands; CPC: Collection of P.W. Crous; DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; FRC: Fusarium Research Center, Penn State University, Pennsylvania; GJS: Collection of Gary J. Samuels; IHEM: Institute of Hygiene and Epidemiology-Mycology Laboratory, Brussels, Belgium; MRC: National Research Institute for Nutritional

Diseases, Tygerberg, South Africa; NRRL: Agricultural Research Service Culture Collection, USA; PD: Collection of the Dutch National Plant Protection Organization, Wageningen, The Netherlands. T: Ex-type culture.

References

Carbone, I., and Kohn, L. M. 2019. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91:553–556.

Catti, A., Pasquali, M., Ghiringhelli, D., Garibaldi, A., and Gullino, M. L. 2007. Analysis of Vegetative Compatibility Groups of *Fusarium oxysporum* from *Eruca vesicaria* and *Diplotaxis tenuifolia*. J. Phytopathol. 155:61–64.

Claerbout J., Van Poucke K., Mestdagh H., Delaere I., Vandevelde I., Venneman S., Decombel A., Bleyaert P., Neukermans J., Viaene N., Heungens K., Höfte M. (2023) *Fusarium* isolates from Belgium causing wilt in lettuce show genetic and pathogenic diversity. Plant Pathology 72, 593-609.

Claerbout, J., Venneman, S., Vandevelde, I., Decombel, A., Bleyaert, P., Volckaert, A., Neukermans, J., and Höfte, M. 2018. First Report of *Fusarium oxysporum* f. sp. *lactucae* Race 4 on Lettuce in Belgium. Plant Dis. 10:1037.

Edwards, J., Auer, D., de Alwis, S. K., Summerell, B., Aoki, T., Proctor, R. H., Busman, M., and O'Donnell, K. 2016. *Fusarium agapanthi* sp. nov., a novel bikaverin and fusarubin-producing leaf and stem spot pathogen of *Agapanthus praecox* (African lily) from Australia and Italy. Mycologia. 108:981–992.

Franco Ortega, S., Tomlinson, J., Gilardi, G., Spadaro, D., Gullino, M. L., Garibaldi, A., Boonham, N. 2018. Rapid detection of *Fusarium oxysporum* f. sp. *lactucae* on soil, lettuce seeds and plants using loop-mediated isothermal amplification. Plant Pathol. 67:1462–1473.

Fujinaga, M., Ogiso, H., Shinohara, H., Tsushima, S., Nishimura, N., Togawa, M., Saito, H., and Nozue, M. 2005. Phylogenetic relationships between the lettuce root rot pathogen *Fusarium oxysporum* f. sp. *lactucae* races 1, 2, and 3 based on the sequence of the intergenic spacer region of its ribosomal DNA. J. Gen. Plant Pathol. 71:402–407.

Fujinaga, M., Ogiso, H., Tuchiya, N., Saito, H., Yamanaka, S., Nozue, M., and Kojima, M. 2003. Race 3, a new race of *Fusarium oxysporum* f. sp. *lactucae* determined by a differential system with commercial cultivars. J. Gen. Plant Pathol. 69:23–28.

Fujinaga, M., Ogiso, H., Tsuchiya, N., and Saito, H. 2001. Physiological Specialization of *Fusarium oxysporum* f. sp. *lactucae*, a Causal Organism of Fusarium Root Rot of Crisp Head Lettuce in Japan. J. Gen. Plant Pathol. 67:205–206.

Galvez L., Brizuela A.M., Garces I., Cainarca J.S., Palmero D. (2023) First report of *Fusarium oxysporum* f. sp. *lactucae* race 4 causing lettuce wilt in Spain. Plant Disease 107, 2549-2550.

Garibaldi, A., Gilardi, G., and Gullino, M. L. 2006. Evidence for an expanded host range of *Fusarium oxysporum* f. sp. *raphani*. Phytoparasitica. 34:115–121 Available at: https://link.springer.com/article/10.1007/BF02981311 [Accessed August 19, 2023].

Garibaldi, A., Gilardi, G., and Gullino, M. L. 2004b. First Report of *Fusarium oxysporum* Causing Vascular Wilt of Lamb's Lettuce (*Valerianella olitoria*) in Italy. Plant Dis. 88:83–83.

Garibaldi, A., Gilardi, G., and Gullino, M. L. 2004a. Varietal resistance of lettuce to *Fusarium oxysporum* f. sp. *lactucae*. Crop Protect. 23:845–851.

Garibaldi, A., Gilardi, G., and Gullino, M. L. 2002. First Report of *Fusarium oxysporum* on Lettuce in Europe. Plant Dis. 86:1052–1052.

Geiser, D. M., Aoki, T., Bacon, C. W., Baker, S. E., Bhattacharyya, M. K., Brandt, M. E., ... & Zhang, N. 2013. One fungus, one name: defining the genus Fusarium in a scientifically robust way that preserves longstanding use. Phytopathol. 103: 400-408.

Geiser, D. M., Jiménez-Gasco, M. D. M., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T. J., Zhang, N., Kuldau, G. A., and O' Donnell, K. 2004.

FUSARIUM-ID v. 1.0: A DNA sequence database for identifying Fusarium. Eur. J. Plant Pathol. 110:473–479.

Gilardi, G., Vasileiadou, A., Garibaldi, A., and Gullino, M. L. 2021. Low temperatures favour Fusarium wilt development by race 4 of *Fusarium oxysporum* f. sp. *lactucae*. J. Plant Pathol. 103:973–979.

Gilardi, G., Garibaldi, A., Matic, S., Senatore, M. T., Pipponzi, S., Prodi, A., and Gullino, M. L. 2019. First Report of *Fusarium oxysporum* f. sp. *lactucae* Race 4 on Lettuce in Italy. Plant Dis. 103:2680-2680.

Gilardi, G., Franco Ortega, S., van Rijswick, P. C. J., Ortu, G., Gullino, M. L., and Garibaldi, A. 2017b. A new race of *Fusarium oxysporum* f. sp. *lactucae* of lettuce. Plant Pathol. 66:677–688.

Gilardi, G., Pons, C., Gard, B., Franco-Ortega, S., and Gullino, M. L. 2017a. Presence of Fusarium Wilt, Incited by *Fusarium oxysporum* f. sp. *lactucae*, on Lettuce in France. Plant Dis. 101:1053.

Gilardi, G., Sendhilvel, V., Garibaldi, A., and Gullino, M. L. 2008. Lamb's lettuce (*Valerianella olitoria*): New host of *Fusarium oxysporum* f. sp. *conglutinans*. J. Plant Dis. Protect. 115:229–233.

Gordon, T. R., and Koike, S. T. 2015. Management of Fusarium wilt of lettuce. Crop Prot. 73: 45-49.

Groenewald, J. Z., Nakashima, C., Nishikawa, J., Shin, H. D., Park, J. H., Jama, A. N., Groenewald, M., Braun, U., and Crous, P. W. 2013. Species concepts in Cercospora: spotting the weeds among the roses. Stud. Mycol. 75:115–170.

Guerrero, M. M., Martínez, M. C., León, M., Armengol, J., and Monserrat, A. 2020. First Report of Fusarium Wilt of Lettuce Caused by *Fusarium oxysporum* f. sp. *lactucae* Race 1 in Spain. Plant Dis. 104:1858-1858.

Gullino M.L., Gilardi G., Garibaldi A. 2019. Ready-to-Eat Salad Crops: A Plant Pathogen's Heaven. Plant Dis. 103: 2053-2070.

Gullino, M. L., Gilardi, G., and Garibaldi, A. 2004. New Fusarium wilts on vegetable crops in Italy. Commun. Agric. Appl. Biol. Sci. 69:405–13.

Herrero, M. L., Nagy, N. E., and Solheim, H. 2021. First Report of *Fusarium oxysporum* f. sp. *lactucae* Race 1 Causing Fusarium Wilt of Lettuce in Norway. Plant Dis.105: 2239.

Hillis, D. M., and Bull, J. J. 1993. An Empirical Test of Bootstrapping as a Method for Assessing Confidence in Phylogenetic Analysis. Syst. Biol. 42:182–192.

Huang J. H, and Lo, C. T. 1998. Wilt of lettuce caused by *Fusarium oxysporum* in Taiwan. Plant Pathol. Bull. 7:150–153.

Hubbard J.C, and Gerik J. S. 1993. A New Wilt Disease of Lettuce Incited by *Fusarium oxysporum* f. sp. lactucum *forma specialis* nov. Plant Dis. 77:750.

ISTAT. 2022. http://dati.istat.it/Index.aspx?QueryId=33703&lang=en. Accessed August 19, 2023.

Katoh, K., and Standley, D. M. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30:772–780.

Komada, H. 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Rev. Plant Prot. Res. 8:114-124.

Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33:1870–1874.

Leslie, J. F., Summerell, B. A. 2006. The Fusarium Laboratory Manual, 1st ed.; Blackwell Publishing: Ames, IA, USA.

Lin, Y. H., Lai, P. J., Chang, T. H., Wan, Y. L., Huang, J. W., Huang, J. H., and Chang, P. F. L. 2014. Genetic diversity and identification of race 3 of *Fusarium oxysporum* f. sp. *lactucae* in Taiwan. Eur. J. Plant Pathol. 140:721–733.

Liu, Y. J., Whelen, S., and Hall, B. D. 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. Mol. Biol. Evol. 16:1799–1808.

Llorach, R., Martínez-Sánchez, A., Tomás-Barberán, F. A., Gil, M. I., and Ferreres, F. 2008. Characterisation of polyphenols and antioxidant properties of five lettuce varieties and escarole. Food Chem. 108:1028–1038.

Lombard, L., Sandoval-Denis, M., Lamprecht, S. C., and Crous, P. W. 2019. Epitypification of *Fusarium oxysporum* - clearing the taxonomic chaos. Persoonia. 43:1–47.

Lombard, L., van der Merwe, N. A., Groenewald, J. Z., and Crous, P. W. 2015. Generic concepts in Nectriaceae. Stud. Mycol. 80:189–245.

Marques Ramalhete M. de L., Gonçalves Bastos C., Matias Furtado A. J. 2006 [New diseases of lettuce in the region of Entre Douro e Minho]. O Minho, a Terra e o Homem, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, pp 61-65.

Matheron, M. E., McCreight, J. D., Tickes, B. R., and Porchas, M. 2005. Effect of Planting Date, Cultivar, and Stage of Plant Development on Incidence of Fusarium Wilt of Lettuce in Desert Production Fields. Plant Dis. 89:565–570.

Matuo, T., and Motohashi, S. 1967. On *Fusarium oxysporum* f. sp. *lactucae* n. f. causing root rot of lettuce. Trans. Mater. Res. Soc. Jpn. 8:13–5.

Mbofung, G. Y., Hong, S. G., and Pryor, B. M. 2007. Phylogeny of *Fusarium oxysporum* f. sp. *lactucae* Inferred from Mitochondrial Small Subunit, Elongation Factor 1-alpha, and Nuclear Ribosomal Intergenic Spacer Sequence Data. Phytopathology 97:87–98 .

Millani, M. J., Etebarian, H., and Alizadeh, A. 1999. Occurrence of Fusarium wilt of lettuce in Shahr-ray, Varamin and Karaj areas. Iran. J. Plant Pathol. 35:44–45.

Motohashi, S., Abe, Z., and Ogawa, T. 1960. Occurrence of lettuce root rot. Ann. Phytopathol. Soc. Jpn. 25:47

Nylander, J. A. A. 2004. MrModeltest V2. Program Distributed by the Author. Evolutionary Biology Centre, Uppsala University, Uppsala.

O'Donnell, K., Ward, T. J., Robert, V. A. R. G., Crous, P. W., Geiser, D. M., and Kang, S. 2015. DNA sequence-based identification of *Fusarium*: Current status and future directions. Phytoparasitica 43:583–595.

O'Donnell, K., Sutton, D. A., Rinaldi, M. G., Sarver, B. A. J., Balajee, S. A., Schroers, H. J., Summerbell, R.C., Robert, V.A., Crous, P.W., Zhang, N., and Aoki, T. 2010. Internet-accessible DNA sequence database for identifying fusaria from human and animal infections. J. Clin. Microbiol. 48:3708–3718.

O'Donnell, K., Sutton, D. A., Rinaldi, M. G., Gueidan, C., Crous, P. W., and Geiser, D. M. 2009. Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-F. equiseti* and *F. chlamydosporum* species complexes within the United States. J Clin Microbiol. 47:3851–3861.

O'Donnell, K., Sutton, D. A., Fothergill, A., McCarthy, D., Rinaldi, M. G., Brandt, M. E., Zhang, N., and Geiser, D. M. 2008. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. J. Clin. Microbiol. 46:2477–2490.

O'Donnell, K., Kistler, H. C., Tacke, B. K., and Casper, H. H. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proc. Natl. Acad. Sci. U S A. 97:7905–7910.

O'Donnell, K., Kistlerr, H. C., Cigelnik, E., and Ploetz, R. C. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana:

concordant evidence from nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci. U S A. 95:2044–2049.

O'Donnell, K., and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylogenet. Evol. 7:103–116.

Pasquali, M., Dematheis, F., Gullino, M. L., and Garibaldi, A. 2007. Identification of Race 1 of *Fusarium oxysporum* f. sp. *lactucae* on Lettuce by Inter-Retrotransposon Sequence-Characterized Amplified Region Technique. Phytopathology. 97:987–996.

Paugh, K. R. and Gordon, T. R. 2019. Effect of planting date and inoculum density on severity of Fusarium wilt of lettuce in California. Plant Dis. 103: 1498–1506.

Ronquist, F., Teslenko, M., Van der Mark, P. Ayres, D. L. Darling, A. Höhna, S. Larget, B. Liu, L. Suchard, M. A., and Huelsenbeck, J. P. 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61:539-542.

Scott J. C., McRoberts, D. N., Gordon, T. R. 2014. Colonization of lettuce cultivars and rotation crops by *Fusarium oxysporum* f. sp. *lactucae*, the cause of Fusarium wilt of lettuce. Plant Pathol., 63 (2014), pp. 548-553

Scott, J. C., Gordon, T. R., Shaw, D. V., and Koike, S. T. 2010. Effect of Temperature on Severity of Fusarium Wilt of Lettuce Caused by *Fusarium oxysporum* f. sp. *lactucae*. Plant Dis. 94:13–17.

Shaner, G., and Finney, R. E. 1977. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 67:1051-1056.

Spadaro, D., and Gullino, M. L. 2022. Crosstalk between wilt-causing fungi, plants and their microbiome. In: Microbial Cross-talk in the Rhizosphere.

Rhizosphere Biology (Horwitz B. A., Mukherjee P. K., eds.), Springer, Singapore. 155-178. DOI: 10.1007/978-981-16-9507-0_7.

Srinivasan, K., Gilardi, G., Spadaro, D., Garibaldi, A., and Gullino, M. L. 2010. Molecular characterization through IGS sequencing of *formae speciales* of *Fusarium oxysporum* pathogenic on lamb's lettuce. Phytopathol. Mediterr. 49:309–320.

Srinivasan, K., Spadaro, D., Poli, A., Gilardi, G., Gullino, M.L., and Garibaldi, A. 2012. Genetic diversity and pathogenicity of *Fusarium oxysporum* isolated from wilted rocket plants in Italy. Phytoparasitica 40:157-170.

Subbarao, K. V., Davis, R. M., Gilbertson, R. L., and Raid, R. N. 2017. Compendium of Lettuce Diseases and Pests, Second Edition. The American Phytopathological Society, St. Paul, MN USA. 165 pp.

Sung, G. H., Sung, J. M., Hywel-Jones, N. L., and Spatafora, J. W. 2007. A multi-gene phylogeny of Clavicipitaceae (Ascomycota, Fungi): Identification of localized incongruence using a combinational bootstrap approach. Mol. Phylogenet. Evol. 44:1204–1223.

Swofford, D. L. 2003. PAUP* Phylogenetic Analysis Using Parsimony, (*and Other Methods); Version 4.0 b10; Sinauer Associates: Sunderland, MA, USA.

Taylor A., Jackson A.C., Clarkson J.P. (2019) First report of *Fusarium oxysporum* f. sp. *lactucae* Race 4 causing lettuce wilt in England and Ireland. Plant Disease, 103, 1033.

Torbati, M., Arzanlou, M., Sandoval-Denis, M., and Crous, P. W. 2019. Multigene phylogeny reveals new fungicolous species in the *Fusarium tricinctum* species complex and novel hosts in the genus *Fusarium* from Iran. Mycol. Prog. 18:119–133.

Tziros, G. T., and Karaoglanidis, G. S. 2023. Identification of *Fusarium oxysporum* f. sp. *lactucae* Race 1 as the Causal Agent of Lettuce Fusarium Wilt in

Greece, Commercial Cultivars' Susceptibility, and Temporal Expression of Defense-Related Genes. Microorganisms 11:1082.

Van Amsterdam, S., Jenkins, S., Clarkson, J. 2023. First report of *Fusarium oxysporum* f. sp. *lactucae* Race 1 causing lettuce wilt in Northern Ireland. Plant Disease 107, 2524.

Ventura, J. A., and Costa, H. 2008. Fusarium Wilt Caused by *Fusarium oxysporum* on Lettuce in Espirito Santo, Brazil. Plant Dis. 92:976.

Woudenberg, J. H. C., Aveskamp, M. M., de Gruyter, J., Spiers, A. G., and Crous, P. W. 2009. Multiple Didymella teleomorphs are linked to the *Phoma clematidina* morphotype. Persoonia. 22:56.

Chapter 6

General Discussion

The kingdom of fungi is ubiquitous and one of the most diverse lineages of eukaryotes that consists of 2.2 to 3.8 million species, with about 2 to 3 million of these species remaining undiscovered (Hawksworth et al., 2017; Hyde et al., 2020; Sun et al., 2020). Fungi formed saprotrophic, parasitic, mutualistic, or endophytic relationship with plants (Jayawardena et al., 2020). Fungal plant pathogens are foremost biotic factors that can cause significant economic and ecological damage to the crops, leading to severe consequences for natural ecosystems (Doehlemann et al., 2017; Hyde et al., 2018). Fungal plant pathogens are predominantly found in phylum Ascomycota, particular to various classes including Sordariomycetes (e.g., *Diaporthe*, *Fusarium* spp.), Dothideomycetes (e.g., *Alternaria*, *Cladosporium* spp.) (Balendres et al., 2023; Wijayawardene et al., 2022).

This PhD thesis demonstrates the original examples of morpho-molecular characterization and pathogenicity of fungal plant pathogens. The presented studies report the correct identification of fungal diseases of fruits and vegetables of economic importance in Piedmont, Italy, such as black rot of sweet cherries caused by *Alternaria* spp., nut rot of hazelnut nut defects caused by *Diaporthe* spp., and Fusarium wilt of lettuce caused by *F. oxysporum* f. sp. *lactucae* that can lead to significant losses in quality, quantity, and yield of essential fruits and vegetables.

Identification and Morphological Characterization

About two decades ago, the identification of fungal species was primarily based on their morphology which could result in incorrect identification due to phenotypic plasticity (Matute and Sepúlveda, 2019; Wijayawardene et al., 2021). Many important pathogenic genera such as *Alternaria*, *Diaporthe* and *Fusarium* couldn't be identified only based on morphological characteristics due to overlapping

morphology (Crous et al., 2015; Maharachchikumbura et al., 2021). Regardless of these limitations, morphological based identification methods are the fastest way to identify the isolates at genus level (Bhunjun et al., 2021).

In this study, 40 isolates of *Alternaria* and 160 isolates of *Diaporthe* were isolated and identified from symptomatic sweet cherry fruits and defected hazelnut nuts respectively. The isolates of *Alternaria* and *Diaporthe* were studied at genus level based on morphological characteristics. Colonies of *Alternaria* isolates were dark grayish with white margins and formed aerial mycelium on PDA, with an average size of conidia (19.7–21.7 × 10.8–11.6) on PCA. While colonies of *Diaporthe* isolates were white to grey and turn brown gradually. They formed aerial mycelium on PDA, and average size of conidia was 8-14.5 x 2-4.9 μm on PNA.

Molecular Characterization

Molecular phylogenetic approaches have proven to be valuable tools for clarifying the species boundaries within various fungal genera (James et al., 2020; Senanayake et al., 2020). However, they also have many limitations during identification of species (Crous et al., 2015). For example, ITS region has been recognized as the universal fungal barcode due to its simple amplification and wide applicability throughout the kingdom fungi (Rajeshkumar et al., 2019; Yin et al., 2017). But it does not work well for those genera which are rich in species e.g., *Alternaria, Fusarium* etc., so it is often limited to genus level identification (Gautam et al., 2022; Lücking et al., 2020; Stielow et al., 2015). Furthermore, the presence of unidentified ITS sequences in GenBank, which may decrease the authenticity of the data and lead to the significant challenges for the correct species identification (Bhunjun et al., 2020; Raja et al., 2017). Many pathogenic genera don't have any ex-type sequences (Cai et al., 2009; Chethana et al., 2021). Due to above mentioned limitations of ITS region, there is need of additional markers such as *rpb2*, *tub2*, *cmda*, *tef-1α* which are successfully used for the correct species delineation (Adeniyi

et al., 2018; Raja et al., 2017). *tef-1α* and *rpb2* have high delineation power, and most used markers especially in *Fusarium* genus. Previous reports showed that *tef-1α* provides good resolution power to the majority of the species while *rpb2* helps to distinguish between closely related species (Lombard et al., 2019). However, identification of fungal species based only on single marker may not reliable and sufficient to discriminate the fungal species due to lower statistical support (Lombard et al., 2019; Manzar et al., 2022).

Therefore, it is recommended to use combined DNA sequences of ribosomal, mitochondrial, and nuclear protein coding regions for the accurate determination of phylogenetic relationships among taxa (Crous et al., 2015; Kanda et al., 2015; Udayanga et al., 2012). The multi-locus phylogeny provides the greater statistical support (minimum bootstrap value 70%, posterior probabilities 0.90), which helps in the establishment of novel taxa (Hu et al., 2023). Maximum likelihood, maximum parsimony, bayesian inference have been used for the identification of pathogenic genera at species level (Rathnayaka et al., 2023). Moreover, DNA sequence data and Polyphasic approach based on morphology have been extensively used for accurate delineation of species (Manawasinghe et al., 2021).

In this study, multi-locus phylogenetic analysis was conducted for the identification of studied fungal pathogens at the species level. Considering the individual gene locus, tree topology and assignment of species were different for some isolates. However, multi-gene phylogeny of four loci Alt-a1, endoPG, OPA 10-2, and *rpb2* allowed us to identify 33 strains as members of *Alternaria arborescens* species complex (AASC), and 7 as members of the *A. alternata*.

As discussed earlier, ITS region alone is not reliable for the identification of Diaporthaceae due to the significant intraspecific variation. Therefore, multi-locus phylogeny approach was used for the identification of *Diaporthe* isolates at species level. Based on morphological characterization and multi-locus phylogenetic

analysis of the ITS, *tef1- α*, and *tub2*, seven *Diaporthe* species were identified, as *D. eres*, *D. foeniculina*, *D. novem*, *D. oncostoma*, *D. ravennica*, *D. rudis*, and *D. sojae*. Based on multi-locus phylogenetic analysis of four loci *rpb2*, *tef-1α*, *cmdA* and *tub2* FOL race 1 and 4 were identified as *F. curvatum*, FOL race 2 identified as *F. curvatum* and *F. odoratissimum*, while FOL race 3 identified as *F. cugenangense*. Our phylogeny results showed that there was a significant genetic variation present between isolates of the four races of FOL.

Pathogenicity test

The emergence and progression of the plant disease depends on the tripartite interaction of the pathogen, host, and environment (De Wolf and Isard, 2007; Velásquez et al., 2018). Therefore, correct identification of diseases and understanding of these factors along with pathogenicity play a crucial role in early detection of the causative pathogens and global security by preventing the spread of the disease (Dayarathne et al., 2020; Jayawardena et al., 2020). In order to identify the cause of disease it is important to not only identify the fungal species correctly but also confirm whether the organism can cause damage to the host or not (Azuddin et al., 2022; Olszak-Przybyś et al., 2023). This is important because numerous organisms can be associated with fruits as epiphytes or endophytes, without causing any disease. Moreover, endophytes have the potential to become latent pathogens under specific environmental conditions, but when the host matures, they cause diseases (Kusari et al., 2014; Sarsaiya et al., 2020). It is therefore necessary to ensure that correct isolate was obtained from observed symptoms by inoculating the host with all isolates (Bhunjun et al., 2021; Senanayake et al., 2020).

In our pathogenicity test, sweet cherry fruits were wounded and inoculated with conidial suspension of *Alternaria* strains. Pathogenicity results showed that all the *Alternaria* strains were pathogenic, had high virulence, and produced irregular lesions when fruits were inoculated by conidial suspension.

Regarding the second study of this thesis, pathogenicity test was performed on hazelnut nuts 'Tonda Gentile del Piemonte' using a mycelium plug. The pathogenicity tests showed that all the studied isolates were pathogenic on hazelnut nuts, resulting in different disease severity. Moreover, *D. eres*, *D. foeniculina*, *D. novem*, *D. oncostoma*, *D. ravennica*, *D. rudis*, and *D. sojae* were able to cause nut rot of hazelnut. *Diaporthe eres* was the most virulent species as compared to the others.

Considering the study on *Fusarium* spp., pathogenicity was performed on five species to confirm the Koch's postulates: lettuce cv. Romana Verde, lamb's lettuce cv. Trophy, spinach cv. Zebu, wild rocket cv. Winter and cultivated rocket cv. Coltivata. Pathogenicity tests showed that most isolates were pathogenic on lettuce with different levels of virulence; in particular, isolates of races 3 and 4 showed a higher level of virulence. Isolates of FOL race 3 and 4 were pathogenic not only on lettuce but also on lamb's lettuce. The races 1 and 4 were identified through pathogenicity test with a set of differential lettuce cultivars for some isolates.

General conclusion

Fungal taxonomy has entered a new stage with methodological and conceptual progress. The adoption of a single, universal approach for the identification of fungi proves challenging due to the different requirements by communities that focus on different groups, and most of the species remain undocumented (Lücking et al., 2021). A polyphasic approach is needed for the accurate identification of pathogenic fungal species especially when new species causing plant diseases are introduced (Jayawardena et al., 2021). Furthermore, careful combined analysis of biological, ecological, morphological and phylogenetic data is crucial for establishing stable nomenclature, facilitating effective communication regarding plant diseases (Aime et al., 2021). Therefore, accurate identification, distribution and naming of different fungal pathogens (*Alternaria*, *Diaporthe*, *Neofusicoccum* and *Fusarium*) in an area

is essential for the correct and effective management strategies, thus minimizing the production yield loss.

References

Adeniyi, M., Titilawo, Y., Oluduro, A., Odeyemi, O., Nakin, M., & Okoh, A. I. (2018). Molecular identification of some wild Nigerian mushrooms using internal transcribed spacer: polymerase chain reaction. AMB Express, 8(1), 1-9. doi: 10.1186/s13568-018-0661-9.

Aime, M. C., Miller, A. N., Aoki, T., Bensch, K., Cai, L., Crous, P. W., ... & Schoch, C. L. (2021). How to publish a new fungal species, or name, version 3.0. IMA fungus, 12, 1-15. doi: 10.1186/s43008-021-00063-1.

Azuddin, N. F., Mohd, M. H., Nik Rosely, N. F., Mansor, A., & Zakaria, L. (2022). Evaluation of the pathogenicity of endophytic fungi isolated from spines of rattan (*Calamus castaneus*) against other plant hosts. Journal of Applied Microbiology, 133(5), 3228-3238. doi: 10.1111/jam.15777.

Balendres, M. A. O. (2023). Plant diseases caused by fungi in the Philippines. Mycology in the Tropics, 163-188. doi: 10.1016/B978-0-323-99489-7.00014-7.

Bhunjun, C. S., Phillips, A. J., Jayawardena, R. S., Promputtha, I., & Hyde, K. D. (2021). Importance of molecular data to identify fungal plant pathogens and guidelines for pathogenicity testing based on Koch's Postulates. Pathogens, 10(9), 1096. doi: 10.3390/pathogens10091096.

Bhunjun, C. S., Dong, Y., Jayawardena, R. S., Jeewon, R., Phukhamsakda, C., Bundhun, D., ... & Sheng, J. (2020). A polyphasic approach to delineate species in *Bipolaris*. Fungal Diversity, 102, 225-256. doi: 10.1007/s13225-020-00446-6.

Cai, L., Hyde, K. D., Taylor, P. W. J., Weir, B., Waller, J., Abang, M. M., ... & Shivas, R. G. (2009). A polyphasic approach for studying *Colletotrichum*. Fungal Diversity, 39(1), 183-204.

Chethana, K. T., Manawasinghe, I. S., Hurdeal, V. G., Bhunjun, C. S., Appadoo, M. A., Gentekaki, E., ... & Hyde, K. D. (2021). What are fungal species and how

to delineate them?. Fungal Diversity, 109(1), 1-25. doi: 10.1007/s13225-021-00483-9.

Crous, P. W., Hawksworth, D. L., & Wingfield, M. J. (2015). Identifying and naming plant-pathogenic fungi: past, present, and future. Annual Review of Phytopathology, 53, 247-267. doi: 10.1146/annurev-phyto-080614-120245.

Dayarathne, M. C., Mridha, A. U., & Wang, Y. (2020). Diagnosis of fungal plant pathogens using conventional and molecular approaches. Diagnostics of Plant Diseases. IntechOpen, pp 1–20. https://doi.org/10.5772/intechopen.94980.

De Wolf, E. D., & Isard, S. A. (2007). Disease cycle approach to plant disease prediction. Annual Review of Phytopathology, 45, 203-220. doi: 10.1146/annurev.phyto.44.070505.143329.

Doehlemann, G., Ökmen, B., Zhu, W., & Sharon, A. (2017). Plant pathogenic fungi. Microbiology spectrum, 5(1), 5-1. doi: 10.1128/microbiolspec.funk-0023-2016.

Gautam, A. K., Verma, R. K., Avasthi, S., Sushma, Bohra, Y., Devadatha, B., ... & Suwannarach, N. (2022). Current insight into traditional and modern methods in fungal diversity estimates. Journal of Fungi, 8(3), 226. doi: 10.3390/jof8030226.

Hawksworth, D. L., & Lücking, R. (2017). Fungal diversity revisited: 2.2 to 3.8 million species. Microbiology spectrum, 5(4), 10-1128. doi: 10.1128/microbiolspec.funk-0052-2016.

Hu, Y. F., Liu, J. W., Luo, X. X., Xu, Z. H., Xia, J. W., Zhang, X. G., ... & Ma, J. (2023). Multi-locus phylogenetic analyses reveal eight novel species of *Distoseptispora* from southern China. Microbiology Spectrum, e02468-23. doi: 10.1128/spectrum.02468-23.

Hyde, K. D., Al-Hatmi, A. M., Andersen, B., Boekhout, T., Buzina, W., Dawson, T. L., ... & Tsui, C. K. (2018). The world's ten most feared fungi. Fungal Diversity, 93, 161-194. doi: 10.1007/s13225-018-0413-9.

Hyde, K. D., Jeewon, R., Chen, Y. J., Bhunjun, C. S., Calabon, M. S., Jiang, H. B., ... & Lumyong, S. (2020). The numbers of fungi: is the descriptive curve flattening?. Fungal Diversity, 103, 219-271. doi: 10.1007/s13225-020-00458-2.

James, T. Y., Stajich, J. E., Hittinger, C. T., & Rokas, A. (2020). Toward a fully resolved fungal tree of life. Annual Review of Microbiology, 74, 291-313. doi: 10.1146/annurev-micro-022020-051835. doi: 10.1146/annurev-micro-022020-051835.

Jayawardena, R. S., Hyde, K. D., de Farias, A. R. G., Bhunjun, C. S., Ferdinandez, H. S., Manamgoda, D. S., ... & Thines, M. (2021). What is a species in fungal plant pathogens?. Fungal Diversity, 109(1), 239-266. doi: 10.1007/s13225-021-00484-8.

Jayawardena, R. S., Hyde, K. D., Chen, Y. J., Papp, V., Palla, B., Papp, D., ... & Wang, Y. (2020). One stop shop IV: taxonomic update with molecular phylogeny for important phytopathogenic genera: 76–100 (2020). Fungal Diversity, 103, 87-218. doi: 10.1007/s13225-020-00460-8.

Kanda, K., Pflug, J. M., Sproul, J. S., Dasenko, M. A., & Maddison, D. R. (2015). Successful recovery of nuclear protein-coding genes from small insects in museums using Illumina sequencing. PLoS One, 10(12), e0143929. doi: 10.1371/journal.pone.0143929.

Kusari, P., Spiteller, M., Kayser, O., Kusari, S. (2014). Recent Advances in Research on *Cannabis sativa* L. Endophytes and Their Prospect for the Pharmaceutical Industry. In: Kharwar, R., Upadhyay, R., Dubey, N., Raghuwanshi, R. (eds) Microbial Diversity and Biotechnology in Food Security. Springer, New Delhi. doi: 10.1007/978-81-322-1801-2_1.

Lombard, L., Sandoval-Denis, M., Lamprecht, S. C., & Crous, P. W. (2019). Epitypification of *Fusarium oxysporum*–clearing the taxonomic chaos. Persoonia-Molecular Phylogeny and Evolution of Fungi, 43(1), 1-47. doi: 10.3767/persoonia.2019.43.01.

Lücking, R., Aime, M. C., Robbertse, B., Miller, A. N., Aoki, T., Ariyawansa, H. A., ... & Schoch, C. L. (2021). Fungal taxonomy and sequence-based nomenclature. Nature microbiology, 6(5), 540-548. doi: 10.1038/s41564-021-00888-x.

Lücking, R., Aime, M. C., Robbertse, B., Miller, A. N., Ariyawansa, H. A., Aoki, T., ... & Schoch, C. L. (2020). Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding?. IMA fungus, 11(1), 1-32. doi: 10.1186/s43008-020-00033-z.

Maharachchikumbura, S. S., Chen, Y., Ariyawansa, H. A., Hyde, K. D., Haelewaters, D., Perera, R. H., ... & Stadler, M. (2021). Integrative approaches for species delimitation in Ascomycota. Fungal Diversity, 109(1), 155-179. doi: 10.1007/s13225-021-00486-6.

Manawasinghe, I. S., Phillips, A. J., Xu, J., Balasuriya, A., Hyde, K. D., Stępień, Ł., ... & Cheewangkoon, R. (2021). Defining a species in fungal plant pathology: beyond the species level. Fungal Diversity, 109(1), 267-282. doi: 10.1007/s13225-021-00481-x.

Manzar, N., Kashyap, A. S., Maurya, A., Rajawat, M. V. S., Sharma, P. K., Srivastava, A. K., ... & Singh, H. V. (2022). Multi-gene phylogenetic approach for identification and diversity analysis of *Bipolaris maydis* and *Curvularia lunata* isolates causing foliar blight of *Zea mays*. Journal of Fungi, 8(8), 802. doi: 10.3390/jof8080802.

Matute, D. R., & Sepúlveda, V. E. (2019). Fungal species boundaries in the genomics era. Fungal Genetics and Biology, 131, 103249. doi: 10.1016/j.fgb.2019.103249.

Olszak-Przybyś, H., Korbecka-Glinka, G., & Patkowska, E. (2023). Identification and Pathogenicity of *Fusarium* Isolated from Soybean in Poland. Pathogens, 12(9), 1162. doi: 10.3390/pathogens12091162.

Puvača, N., Bursić, V., Vuković, G., Budakov, D., Petrović, A., Merkuri, J., ... & Cara, M. (2020). Ascomycete fungi (*Alternaria* spp.) characterization as major feed grains pathogens. Journal of Agronomy, Technology and Engineering Management, 3(6), 499-505.

Raja, H. A., Miller, A. N., Pearce, C. J., & Oberlies, N. H. (2017). Fungal identification using molecular tools: a primer for the natural products research community. Journal of natural products, 80(3), 756-770. doi: 10.1021/acs.jnatprod.6b01085.

Rajeshkumar, K. C., Yilmaz, N., Marathe, S. D., & Seifert, K. A. (2019). Morphology and multigene phylogeny of *Talaromyces amyrossmaniae*, a new synnematous species belonging to the section *Trachyspermi* from India. MycoKeys, (45), 41. doi: 10.3897/mycokeys.45.32549.

Rathnayaka, A. R., Chethana, K. T., Phillips, A. J., Liu, J. K., Samarakoon, M. C., Jones, E. G., ... & Zhao, C. L. (2023). Re-evaluating Botryosphaeriales: Ancestral state reconstructions of selected characters and evolution of nutritional modes. Journal of Fungi, 9(2), 184. doi: 10.3390/jof9020184

Sarsaiya, S., Jain, A., Jia, Q., Fan, X., Shu, F., Chen, Z., ... & Chen, J. (2020). Molecular identification of endophytic fungi and their pathogenicity evaluation against *Dendrobium nobile* and *Dendrobium officinale*. International Journal of Molecular Sciences, 21(1), 316. doi: 10.3390/ijms21010316.

Senanayake, I. C., Rathnayaka, A. R., Marasinghe, D. S., Calabon, M. S., Gentekaki, E., Lee, H. B., ... & Xiang, M. M. (2020). Morphological approaches in studying fungi: Collection, examination, isolation, sporulation and

preservation. Mycosphere, 11(1), 2678-2754. doi 10.5943/mycosphere/11/1/20.

Stielow, J. B., Levesque, C. A., Seifert, K. A., Meyer, W., Irinyi, L., Smits, D., ... & Robert, V. (2015). One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. Persoonia-Molecular Phylogeny and Evolution of Fungi, 35(1), 242-263. doi: 10.3767/003158515X689135.

Sun, S., Hoy, M. J., & Heitman, J. (2020). Fungal pathogens. Current Biology, 30(19), R1163-R1169. doi: 10.1016/j.cub.2020.07.032.

Udayanga, D., Liu, X., Crous, P. W., McKenzie, E. H., Chukeatirote, E., & Hyde, K. D. (2012). A multi-locus phylogenetic evaluation of *Diaporthe* (*Phomopsis*). Fungal diversity, 56, 157-171. doi: 10.1007/s13225-012-0190-9.

Velásquez, A. C., Castroverde, C. D. M., & He, S. Y. (2018). Plant–pathogen warfare under changing climate conditions. Current biology, 28(10), R619-R634. doi: 10.1016/j.cub.2018.03.054.

Wijayawardene, N. N., Phillips, A. J., Pereira, D. S., Dai, D. Q., Aptroot, A., Monteiro, J. S., ... & Thines, M. (2022). Forecasting the number of species of asexually reproducing fungi (Ascomycota and Basidiomycota). Fungal Diversity, 114(1), 463-490. doi: 10.1007/s13225-022-00500-5.

Wijayawardene, N. N., Bahram, M., Sánchez-Castro, I., Dai, D. Q., Ariyawansa, K. G., Jayalal, U., ... & Tedersoo, L. (2021). Current insight into culture-dependent and culture-independent methods in discovering Ascomycetous Taxa. Journal of Fungi, 7(9), 703. doi: 10.3390/jof7090703.

Yin, G., Zhang, Y., Pennerman, K. K., Wu, G., Hua, S. S. T., Yu, J., ... & Bennett, J. W. (2017). Characterization of blue mold *Penicillium* species isolated from stored fruits using multiple highly conserved loci. Journal of fungi, 3(1), 12. doi: 10.3390/jof3010012.

Acknowledgement

I would like to express my deepest gratitude to my worthy PhD tutor Prof. **Davide Spadaro**, for his time, scholastic guidance, patience, motivation, providing all required material, and allowing me to achieve benefit freely from his vast knowledge and immense research experience. His immense knowledge, critical review of the proposals, abstracts, and manuscripts, scientific discussions, and kind guidance encouraged me to move forward and will remain thought-provoking for the rest of my life. I could not have imagined having a better advisor and mentor like him. I am also deeply grateful to my PhD co tutor Dr. **Vladimiro Guarnaccia**, for sparing his time when I was in need, and invaluable contributions to my doctoral research. He consistently provided insightful feedback and constructive criticism, challenging me to think critically and refine my approach. I learnt a lot from Vladimiro in these years.

I would like to express my sincere gratitude to Prof. **Angelo Garibaldi**, President of the Centre Agroinnova, for offering invaluable advice throughout the pathogenicity trials. I also wish to thank Dr. **Giovanna Gilardi** for her technical assistance and expertise in conducting the trials in the greenhouse. Her willingness to share her knowledge and time made a significant difference in the quality of my data. I would like to thank **Domenico Bertetti** for his instructions in making accurate and beautiful microscopic pictures.

I wish to thank Ilaria Martino for her invaluable support and sharing her expertise in laboratory techniques throughout my research journey. My sincere appreciation is also owed to **Simona Prencipe** for her guidance and support in data analysis. I would also like to acknowledge all the colleagues and friends in Agroinnova and DISAFA, **Edoardo Piombo**, **Silvia Valente**, **Athina Vasileiadou**, **Wajid Aurangzeb** for all assistance and their moral support during my work.

Last but not the least, I would like to pay profound thanks to my beloved **Mother** and **Father** for allowing me to realize my potential. Their love, affection, and

prayers all along have been the ultimate source of strength for me. I also thank my ever-loving **Brothers** and **Sisters** for their perpetual prayers, love, and support. Finally, how can I miss acknowledge to my wife **Ghazala Munawar**, whose unwavering support, love, and understanding sustained me throughout the challenging journey of completing PhD. Her encouragement and sacrifices have been the cornerstone of my success. To my precious seven-month-old daughter **Umaimah Waqas**, who brought joy and inspiration into my life during the process of manuscript and thesis writing. Your laughter and innocence provided a source of motivation, reminding me of the importance of the work I was undertaking.

Muhammad Waqas

Curriculum vitae

Muhammad Waqas, born on September 5, 1989, in Narowal, Pakistan. He obtained his Bachelor of Science (Honors) in Plant Pathology from Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, in 2012. Following this, he completed a Master of Science (Honors) in Plant Pathology in 2015, with a focus on “Current Status and molecular characterization of potyviruses infecting cucurbits in Gujranwala Division of Pakistan”.

In November 2019, Waqas move to Turin and started his PhD in Biological Sciences and Applied Biotechnologies from University of Turin, Torino, Italy. His research revolves around "Molecular Characterization and Pathogenicity of Emerging Fungal Pathogens of Fruit and Leafy Vegetables," working under the supervision of Prof. Davide Spadaro.

Publications

International Journals (ISI)

Waqas, M., Guarnaccia, V., Bardella. S. & Spadaro, D. (2023). Molecular characterization and pathogenicity of *Diaporthe* species causing nut rot in hazelnut from Italy. Plant Disease, (ja). https://doi.org/10.1094/PDIS-01-23-0168-RE.

Waqas, M., Prencipe, S., Guarnaccia, V., & Spadaro, D. (2023). Molecular Characterization and Pathogenicity of *Alternaria* spp. Associated with Black Rot of Sweet Cherries in Italy. Journal of Fungi, 9(10), 992.

Waqas, M., Guarnaccia, V., & Spadaro, D. (2022). First Report of Nut Rot Caused by *Neofusicoccum parvum* on Hazelnut (*Corylus avellana*) in Italy. Plant Disease, 106(7), 1987.

Kuhn, J. H., Adkins, S., Agwanda, B. R., Al Kubrusli, R., Alkhovsky, S. V., Amarasinghe, G. K., ... & Nowotny, N. (2021). Correction to: 2021 Taxonomic update of phylum Negarnaviricota (Riboviria: Orthornavirae), including the large orders Bunyavirales and Mononegavirales. Archives of virology, 166(12), 3567-3579.

Ashfaq, M., **Waqas, M**., Ahmed, N., Raheel, M., Abbas, H. T., Masroor, A., ... & Alfarraj, S. (2021). Molecular characterization and identification of economically important Potyviruses in Cucurbitaceae family from Gujranwala division of Punjab, Pakistan. Journal of King Saud University-Science, 33(8), 101642.

Liu, H., Wang, G., Yang, Z., Wang, Y., Zhang, Z., Li, L., **Waqas, M**., Zhang, J., Xu, L., Qi, L., Hong, J., & Hong, N. (2020). Identification and Characterization of

a Pear Chlorotic Leaf Spot-associated Virus, a Novel Emaravirus Associated with a Severe Disease of Pear Trees in China. Plant Disease, 104(11), 2786-2798.

Hafeez, M., Qasim, M., Ali, S., Yousaf, H., **Waqas, M**., Ali, E., Ahmad, M., Jan, S., Bashir, M., Noman, A., Wang, M., Gharmh, H. and Khan, K. (2020). Expression and functional analysis of P450 gene induced tolerance/resistance to lambda-cyhalothrin in quercetin fed larvae of beet armyworm *Spodoptera exigua* (Hübner). Saudi journal of biological sciences, 27(1), 77-87.

Rehman, A.U.; Li, Z.; Yang, Z.; **Waqas, M**.; Wang, G.; Xu, W.; Li, F.; Hong, N. (2019). The Coat Protein of Citrus Yellow Vein Clearing Virus Interacts with Viral Movement Proteins and Serves as an RNA Silencing Suppressor. Viruses, 11, 329.

Ashfaq, M., Saleem, A., **Waqas, M**., & Mukhtar, T. (2017). Natural occurrence and host range studies of Cucumber Mosaic Virus (CMV) infecting ornamental species in Rawalpindi-Islamabad area of Pakistan. Philipp. Agric. Scient, 100, 55-61.

Abstracts presented at Congresses

Muhammad Waqas, Giovanna Gilardi, Vladimiro Guarnaccia, Angelo Garibaldi, Davide Spadaro (2023). Different species of *Fusarium* cause vascular wilt of lettuce: molecular and pathogenicity approach. European Fusarium Seminar. Rome, June 12-15, 2023. www.efs16.it

Muhammad Waqas, Allan Pizzinat, Vladimiro Guarnaccia and Davide Spadaro. (2022). Genetic diversity and pathogenicity of *Diaporthe* spp. causing hazelnut defects of hazelnut nuts from Italy. VI International Symposium on Postharvest Pathology: Innovation and Advanced Technologies for Managing Postharvest Pathogens. May 29- June 2, 2022.

Muhammad Waqas, Vladimiro Guarnaccia and Davide Spadaro. 2022. Genetic diversity and pathogenicity of Botryosphaeriaceae and Diaporthaceae causing defects of hazelnut nuts from Italy. 31st Fungal Genetics Conference, the Asilomar Conference Grounds in Pacific Grove, California, USA. March 15-20, 2022.

Muhammad Ashfaq, **Muhammad Waqas**, S. B. Kayani, Nadeem Ahmed, Rana Binyamin, Zohaib Asad, Mirza Abid Mehmood, Hasan Riaz and Muhammad Arslan Khan. 2021. Molecular Characterization and Identification of Economically Important Potyviruses in Cucurbitaceae Family from Gujranwala Division of Punjab, Pakistan. International Conference on Smart Plant Protection (online). January 27-28, 2021. Institute of Plant Protection MNS-University of Agriculture, Multan, Pakistan.

Ashfaq, M., A. Saleem, T. Mukhtar and **M. Waqas**. 2014. The Incidence and distribution of Cucumber mosaic cucumovirus in ornamentals in Islamabad and Rawalpindi, Pakistan. "Intl. Conf. on Stress Biol. & Biotech. Challenges & Management". 21-23 May 2014 IAGS, University of the Punjab, Lahore, Pakistan.

Non-ISI Journals

Waqas, M., Guarnaccia, V., Spadaro, D., & Garibaldi, A. (2022). Genetic diversity and pathogenicity of *Fusarium oxysporum* species complex causing wilt on lettuce. *PROTEZIONE DELLE COLTURE*, *15*(2), 39-39.

Waqas, M., Gilardi, G., Guarnaccia, V., Spadaro, D., & Garibaldi, A. (2021). Genetic diversity and pathogenicity of *Fusarium oxysporum* isolated from different leafy vegetables. *PROTEZIONE DELLE COLTURE*, *14*(2), 55-55.