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Diagnostics, epidemiology and control of emerging oomycetes and phytopathogenic fungi

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Patologia vegetale

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Abstract

Climate warming constitutes a primary threat to the overall health of global forests. The impacts of climate on forest ecosystems are manifold and intricate, encompassing interplays affecting species physiology and phenology, the geographical distribution of vegetation, and biogeochemical cycles. Another consequence of climate change is the modification of ecosystem processes, e.g. the rate of litter decomposition and habitat suitability, changes that can ultimately pose a threat to the survival of species within their native ranges. All these alterations can impact forests directly or indirectly. In fact, they not only weaken trees but also enhance the reproductive success of pathogens: it has been proven that climate anomalies lead to an increased host susceptibility as well as heigthen pathogen virulence, survival rate and biomass (a high pathogen inoculum pressure lead to a higher disease rate on crops).

The problem is further worsened by the introduction of alien, invasive pathogens. In fact, in such a complex scenario, with plants weakened and physiologically impaired, even microorganisms typically considered opportunistic, secondary pathogens turn into aggressive pathogens. The field investigations carried out in this study deal with some severe outbreaks and mortality events that are heavily impacting *Fraxinus* spp. formations in the Mediterranean area. Botryosphaeriaceae and *Phytophthorae* emerged as prominent species in the monitored sites, particularly the fungal pathogens *Botryosphaeria dothidea, Neofusicoccum parvum, Diplodia fraxini* and the oomycetes *P. acerina, P. bilorbang, P. cinnamomi, P. hydropathica, P. lacustris, P. multivora, P. plurivora, P. polonica, P. pseudocryptogea, P. pseudosyringae*, and *P. syringae.*

Botryosphaeriaceae species played a significant role. The characteristic of these fungi is their opportunistic nature, seriously affecting plants when these undergo stress conditions. With ongoing global warming, climatic stressors have become more prominent and frequent, creating greater opportunities for Botryosphaeriaceae to thrive and contribute to the decline of numerous tree species. These pathogens have the capacity to spread across vast areas due to their natural dispersal abilities or can be inadvertently introduced through human activities, impacting the functioning of entire agroecosystems. The epidemic spread of these pathogens is exacerbated by the challenge of promptly identifying the true etiological agent, as various secondary or commensal microorganisms are often associated and complicate the efforts to

identify the true etiologic agents.

To further complicate the issue, pathogens recently introduced into a specific, previously uncontaminated area are often scarcely known and control measures against them are scarce or non-existent. A major drawback in these cases is the lack of effective diagnostic protocols. Reliable and timely diagnosis is in fact essential to promptly identify plant pathogens so that eradication of initial outbreaks can be effectively implemented. Accurate diagnosis is also an unavoidable tool for monitoring campaigns and thus it stands as the basis for the prevention of invasions by harfmul exotic pathogens.

The Italian Peninsula, characterized by a highly variable climatic conditions and high plant diversity, with its position in the centre of the Mediterranean basin, is a crucial crossroad for the exchange of food, vegetables and other plant materials is particularly prone to possible, inadvertent introductions of harmful pathogens. Epidemiological studies in these cases are crucial to evaluate the adaptability of exotic pathogens and the potential threats the pose in new introduction areas. Furthermore, epidemiological studies provide valuable information on the possible strategies to mitigate the impact and the risks the newly introduced disease agents pose to local ecosystems.

In order to prevent possible serious outbreaks in forests, it is imperative to develop new control strategies such as the exploitation of biological control agents (BCAs). Biocontrol agents have a fundamental role in forestry as they can help to manage pathogen attacks without using pesticides. In forest environments, where the use of chemicals could negatively impact the delicate ecological balance (for this reason they are prohibited), sustainable and environmentally-friendly solutions, like those exploiting natural biocontrol organisms, may enable to effectively control harmful pathogens and to preserve the health of forest ecosystems.

Introduction

Forests play a primary role in the functioning of plant communities as they regulate ecosystem functioning, preserve biodiversity, and provide essential goods and services. However, in recent years, forests are under severe threat from the attack of newly introduced pathogens, the resurgence of endemic diseases, and increased stress induced by climate change. The world's climate has always shaped forest structure and diversity. However, today the global climate has become warmer and is changing at an unprecedented rate (Sturrock et al., 2011), causing annual losses of millions of trees and of extensive wooded areas of the planet (Hanewinkel et al., 2013).

The IPCC report emphasizes concerns about global climate change, noting that 2019 saw the highest atmospheric CO_2 concentrations in 2 million years. Furthermore, global surface temperature has increased by 1.09 °C (from 0.95 °C to 1.20 °C) from 2011 to 2020 compared to pre-industrial periods (Legg, 2021). With this trend, climate change is likely to influence the life cycles and biological synchrony of many forest trees and pathogens, leading to changes in the distribution and phenology of events such as budding in host trees, spore release by pathogens, and activities of insects acting as vectors for pathogens. This can significantly alter the incidence and severity of diseases (Sturrock et al., 2011).

The climate change has a dual negative effect. On one hand, it weakens plants, alters their physiology, and predisposes them to attacks from pathogens; on the other hand, it modifies the reproductive and infectious biology of pathogens (especially thermotolerant ones), increasing the frequency of their propagation cycles, hence their inoculum load in the environment, and consequently, the prevalence of disease (Elad and Pertot, 2014).

In Mediterranean countries, over the past four decades, due to an unprecedented increase in travel and international trade, the number of invasive forest pathogens has exponentially increased (Fig. 1) (Garbelotto and Pautasso, 2012; Panzavolta et al., 2021). Invasive exotic species represent the second most significant cause of biodiversity loss and associated ecosystem service depletion, following habitat fragmentation, caused by climate change (Adla et al., 2022). The most severe effects of their spread range from eradicating native plant species to altering the species composition in an area. It is estimated that 37,000 alien

species have been introduced globally through human activities (Roy et al., 2023).

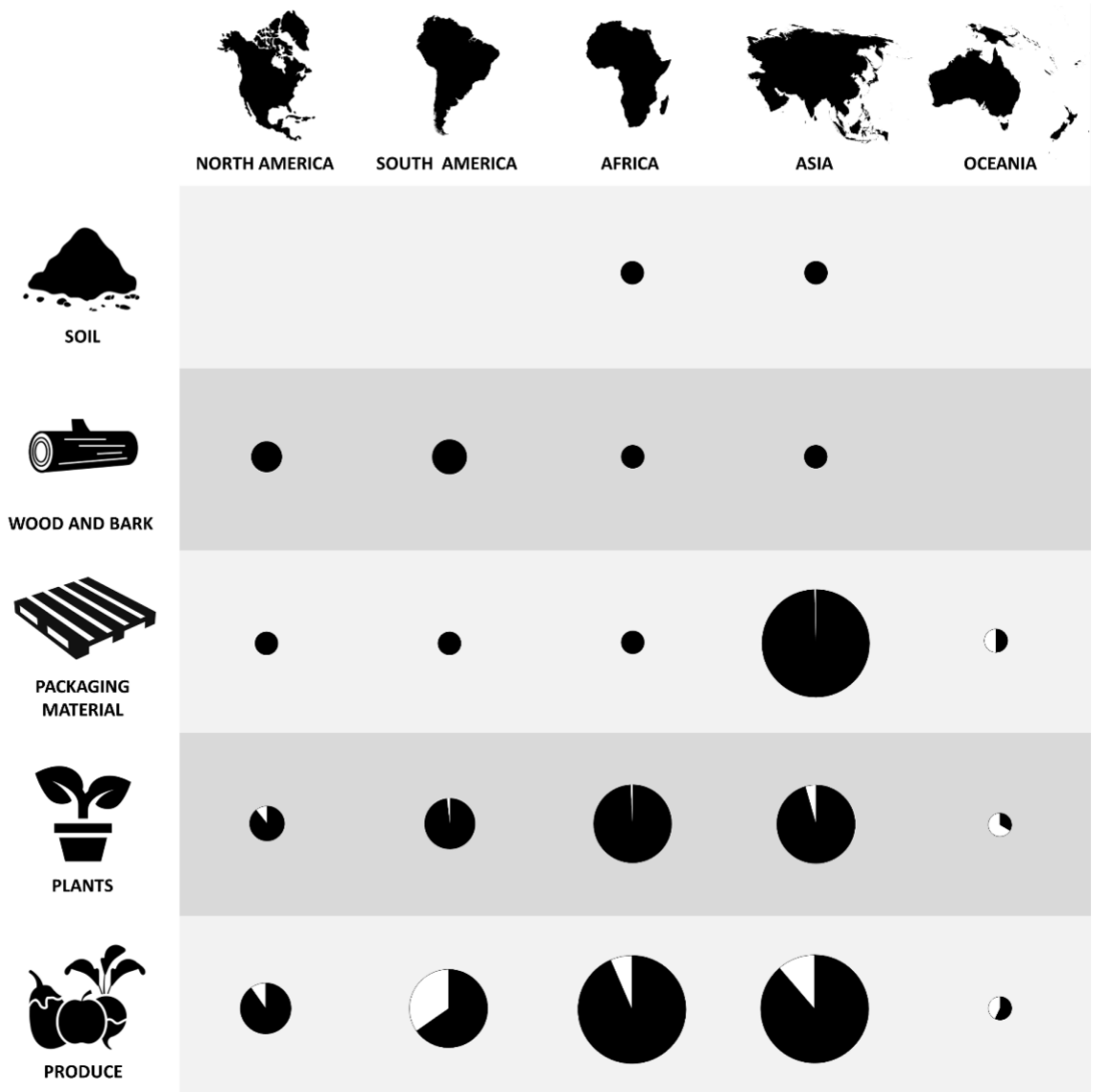

Fig. 1: The frequency of interceptions of fungi and insects at entry ports in Europe during the period 2016-2020 (data obtained from Europhyt reports). The sizes of the circles indicate the relative quantity of interceptions of IAPP (fungi and insects) within each transportation route based on the continents of origin. The black sector of the circle indicates the relative frequency of insect interceptions; the white sector indicates fungal interceptions. Legend: soil = soil and cultivation substrates; wood and bark = roundwood, bark, and processed wood; packaging material = wooden crates, wooden pallets, wooden packaging material, straw; plants = plantation plants, stakes, cuttings, leaves, flowers, foliage-bearing branches; produce = fruit, vegetables, seeds. Credits: Panzavolta et. al., 2021

In Italy, according to the ISPRA database, there are now more than 3,300 invasive species (Roy et al., 2023). Generally, it is the ecological and behavioral flexibility and the

ability to adapt to extreme conditions (temperature, salinity, contaminant concentrations, etc.) that make these species more persistent and less vulnerable compared to local and endemic species. However, their high reproductive capacity, competitiveness, and opportunism can also increase the likelihood of their successful invasion.

The movement of non-native species to new sites can occur either naturally or through human-induced processes, taking advantage of the limited resistance of native communities, a fact that must be ascribed to human disturbance and climate change. Fungal pathogens are moving towards the poles at a rate of 7.61 ± 2.41 km/year in the northern hemisphere, while climates are shifting at 2.7 km/year (Bebber et al., 2013; Marčiulynas et al., 2022).

Among the historical, "emerging" pathogens that have disrupted ecosystems and nearly led to the extinction of important tree species are: *Ophiostoma novo-ulmi*, the causal agent of Dutch elm disease; *Seiridium cardinale*, the pathogen responsible for lethal cypress blight; the root rot agent *Heterobasidion irregulare*, which causes severe attacks to pine, silver fir, Norway spruce, larch, and *Pseudotsuga* sp., as well to beech forests in Calabria (Gonthier and Capretti, 2007); *Hymenoscyphus fraxineus*, responsible for a widespread dieback on *Fraxinus* spp. throughout Europe (Ogris et al., 2010); and *Melampsoridium hiratsukanum*, the rust pathogen which is devastating riparian *Alnus* formations in the Alps (Moricca and Maresi, 2010, Moricca et al., 2021). Moreover, there is particular concern about recent reports of the invasive pathogen *Geosmithia morbida* on *Juglans nigra* and *J. regia* (Moricca et al., 2019; 2020; Bracalini et al., 2023).

Among the several groups of new pathogens, oomycetes have experienced the most significant increase, particularly those belonging to the genus *Phytophthora*, which is one of the most important genera of pathogens causing serious damage with devastating impacts in both agricultural and forest environments (Erwin and Ribeiro, 1996; Jung et al., 2018; Scott et al., 2019; Burgess et al., 2021). These microorganisms, also called "water moulds", can survive for extended periods under adverse conditions, even in preserved structures (Jung et al., 2018), and can exploit the movement of water to infect plants in nurseries, agricultural crops, urban greenery, tree plantations and natural forests (Werres et al., 2007). Some of the most severe *Phytophthora* attacks have been those by *Phytophthora cinnamomi* and *P. cambivora* on *Castanea sativa* (Vannini et al., 2010), *Phytophthora acerina* on *Acer pseudoplatanus* (Ginetti et al., 2014), as well as on various species on *Quercus ilex* and *Q. suber* (Scanu et al., 2013; Linaldeddu et al., 2013) and the devastating attacks of *P. ramorum* on various forest and ornamental species (Rizzo et al., 2002).

In this scenario, a key role is played by certain opportunistic microorganisms known

for their endophytic *habitus* or for living in forest ecosystems as facultative parasites but currently responsible for the severe "decline" of numerous forest species lethal attacks from various weakness pathogens, both root pathogens (*Armillaria, Rosellinia*, etc.) and canker pathogens, such as *Biscogniauxia, Botryosphaeria*, *Diplodia, Sphaeropsis, Phomopsis* (Ragazzi et al., 2004; Benigno et al., 2023).

Members of Botryosphaeriaceae share ecological characteristics with other endophytes of woody plants, displaying diversity, horizontal transmission, spatial structure, and a range of host affinities. Some are aggressive pathogens, causing host death following tree physiology impairment due to various stresses (Benigno et al., 2023; 2024). Their widespread distribution, prolonged latent phase (allowing them to evade quarantine measures), and rapid disease development when hosts are under stress, pose significant threats to agriculture, plantations, and native forests, especially amid climate change crisis. The success of opportunistic endophytes is notable in disturbed environments or non-native plantings. Stressors include water deficit, drought, heat waves, frost, physical damage (e.g. hail), biological stress, competition, and unsuitable sites. Certain Botryosphaeriaceae's lack of host specificity enables them to infect a broad range of hosts, making prevention crucial due to the impracticality of chemical control on a large scale.

Therefore, the phenomenon of forest decline/dieback must be placed in a broad context where many factors interact with each other, with different successions, intensities, and modes, contributing to trigger a series of nonspecific manifestations that individually would not produce the same symptoms.

These factors can be categorized into three groups: Predisposing, inciting and contributing factors (Manion et al., 1981). Predisposing factors compromise the natural host's intrinsic defence mechanisms and increase the susceptibility of trees to long-term damage. These factors are related to climate, site conditions, prolonged periods of drought leading to water stress, soil conditions, forest management practices, and disturbances in land use. Inciting factors may be episodic and of short duration, whether physical or biological. Examples include hailstorms, frost, acute water shortages, and high temperatures. While these factors cause acute, short-term damage with the potential to recover and regress, prolonged and repeated exposure over several years can irreversibly weaken trees, possibly leading to their death. Contributing factors are elements that additionally weaken trees, and when they operate on trees already experiencing physiological impairment, they can be lethal (Fig. 2).

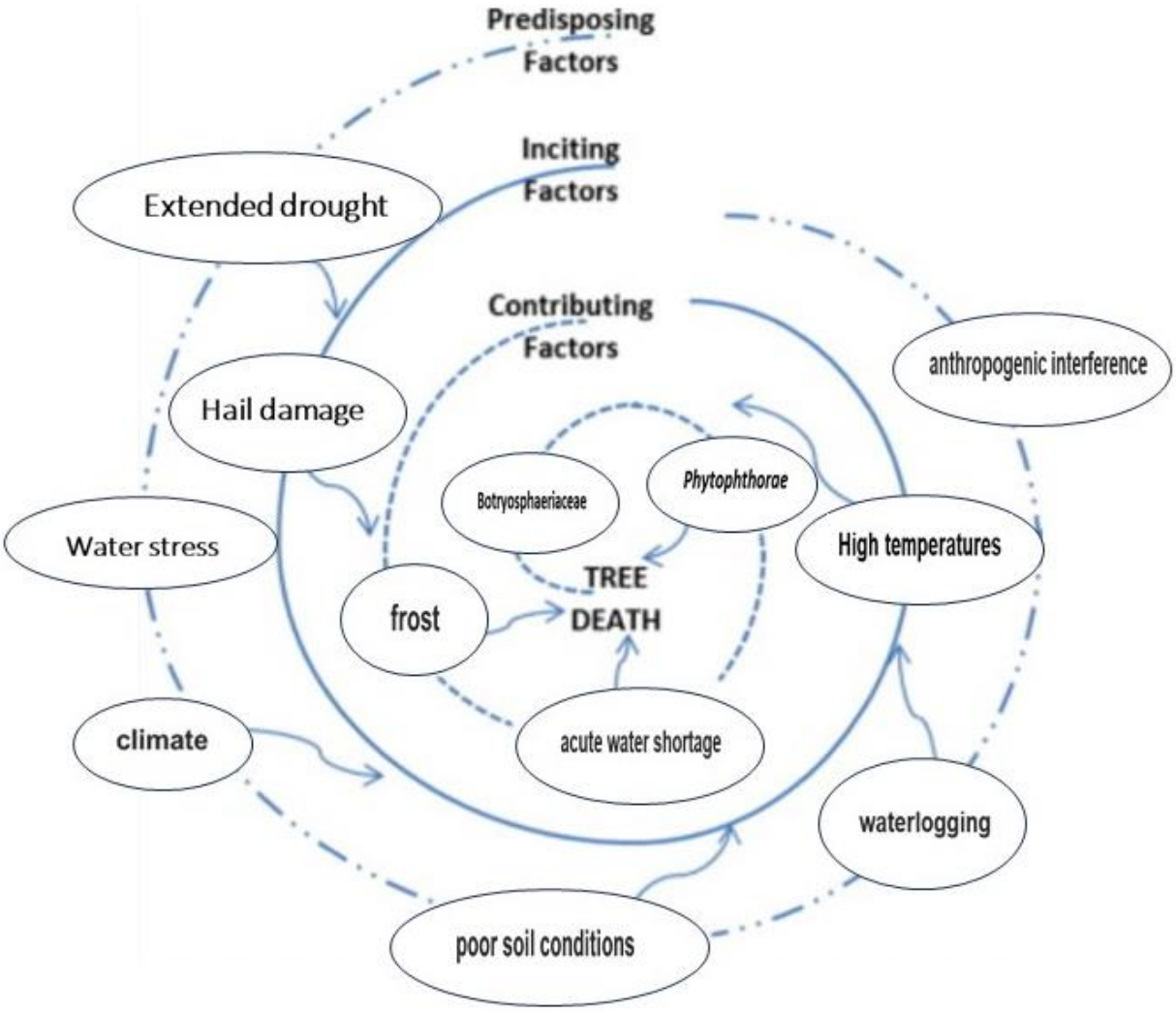

Fig. 2: Adaptation of Manion's (1981) disease spiral with main interacting factors associated with tree decline, classified according to their role in the decline.

Biotic agents that further weaken trees, such as opportunistic pathogens, fall into this category. These pathogens can contribute to the decline and eventual death of already stressed individuals (Manion 1981; Moricca et al., 2008; Panzavolta et al., 2017; Murolo et al., 2021). In fact, pathogens have a broader range of adaptation mechanisms compared to their hosts and shorter generation times, their opportunities for adaptation/evolution are likely to increase. Therefore, it is necessary to develop new strategies to predict and monitor the evolution of plant pathogens in order to prevent the spread of new plant pathogens on vulnerable hosts likely weakened by the pressure of climate change.

In this context, particular attention is directed toward endophytic fungi. There is a lack of consensus within the scientific community regarding the role of endophytes. Closely related to virulent pathogens, but with limited, if any, pathogenic effects themselves, many endophytes

protect host plants from natural enemies (Carrol, 1988; Yan et al., 2019).

In many forest formations affected by decline phenomena ascribed to pathogenic endophytes, oomycetes of the *Phytophthora* genus have often been found associated. In the last 20 years, attention to species of *Phytophthora* into natural ecosystems has significantly increased (Jung, 2009; de Sampaio and Paiva Camilo-Alves, 2013; Hansen, 2015; Mora-Sala et al., 2018; Seddaiu et al., 2020; Benigno et al., 2023; 2024). The progress in understanding the role of these oomycetes depended also on new molecular detection methods that have facilitated the identification and description of new *Phytophthora* species as well as deeper studies of their genetics (Sapkota et al., 2015; Riddell et al., 2019).

Morphological identification, on the other hand, is often problematic also because the unavailability of a specific substrate or antibiotics capable of suppressing the growth of undesidered contaminants, makes it difficult to recover the fungus from environmental samples and, in any case, it requires considerable mycological expertise.

For these reasons, but also due to the increasing global trade (import/export) of plant material, there is a growing demand for accurate and rapid diagnostic protocols to detect plant pathogens from various matrices such as water, soil, air, insects, as well as asymptomatic infected plants, wood, soil, etc (Panzavolta et al., 2021).

Recently, increasingly accurate molecular methods such as PCR and qPCR allow for the timely diagnosis of asymptomatic phytopathogenic microbes (Niessen, 2015; Rizzo et al., 2022). DNA fingerprinting techniques are now considered the most promising and effective diagnostic tool for phytosanitary control of infected material. The ability to detect very small amounts of DNA in a targeted and unequivocal manner makes these techniques an effective and irreplaceable support for both diagnostic and molecular epidemiological applications (Rizzo et al., 2020).

A recent innovation is the loop-mediated isothermal amplification (LAMP) technique, currently used in phytopathological diagnostics. Several protocols have been developed for important pathogens, such as *Dothistroma septosporum* (G. Dorog.) M. Morelet, one of the causal agents of *Dothistroma* needle blight (DNB), an emerging pathogen harmful to natural pine forests. This technology has allowed for the identification of this agent and its differentiation from related pathogens, *Dothistroma pini* Hulbary and *Lecanosticta acicola* (Thüm.) Syd. The LAMP technique has demonstrated superiority over PCR by specifically amplifying target DNA in the presence of non-target sequences, eliminating the need for multiple temperature cycles, long reaction times, and sophisticated laboratory settings that are often responsible for amplification errors and longer response times (Aglietti et al., 2021).

Accurate and timely diagnosis is also essential for understanding the potential impact of parasites on forest ecosystems as well as for implementing effective disease and pest management control strategies (Rizzo et al., 2021; 2022). By using both traditional and molecular approaches, it is possible to identify plant diseases and stop potential outbreaks (Hariharan et al., 2021).

While many efforts have been made in the development of new diagnostic methods, the development of effective control protocols remain the key strategy to contain certain diseases. In forestry, there are few applications of control measures, and to date, the only experiments involve the application of BCAs (BioControl Agents). This strategy in based on the application of beneficial microorganisms – often natural components of forest ecosystems - which can persist and parasitize pathogens. Biocontrol, discovered since the 1920s, has in fact become an increasingly prominent tool in plant protection. By definition, biocontrol involves: a) the destruction of the propagative units or biomass or inoculum of the pathogen; b) the prevention of inoculum formation; c) the weakening or displacement of the pathogen in infected plant residues; d) the reduction of vigour or virulence of the pathogen by agents such as mycoviruses or hypovirulence determinants. Thanks to advances in molecular biotechnology, biological control techniques are now more easily applicable. Control tactics and molecular analyses are fundamental for successfully implementing biocontrol strategies. The efficacy of antagonistic fungi to control fungal pathogens is well-documented. Some BCAs, like some species of *Trichoderma*, a genus of imperfect fungi (Ascomycota division, Hypocreales order) (Gams and Bisset, 1998) are well known for their parasitic properties against various fungal pathogens (their effectiveness having been proven for over 70 years). *Trichoderma* is a natural component of the soil microflora, living also inside plant tissues as an endophyte.

Trichoderma spp., by colonizing the plant without becoming a pathogen, induces a series of metabolic changes in the plant aimed at blocking and/or preventing the entry of the fungal pathogen, confining it to the outer layers of cortical cells (Elad and Kapat, 1999; Elad, 2000). The success of *Trichoderma* species as fungal antagonists stems from their remarkable ability to adapt to unfavorable conditions, high prolificacy, efficiency in nutrient utilization, influence on the rhizosphere, aggressiveness against plant pathogenic fungi, and the capacity to promote plant growth through defence mechanisms (Fig. 3).

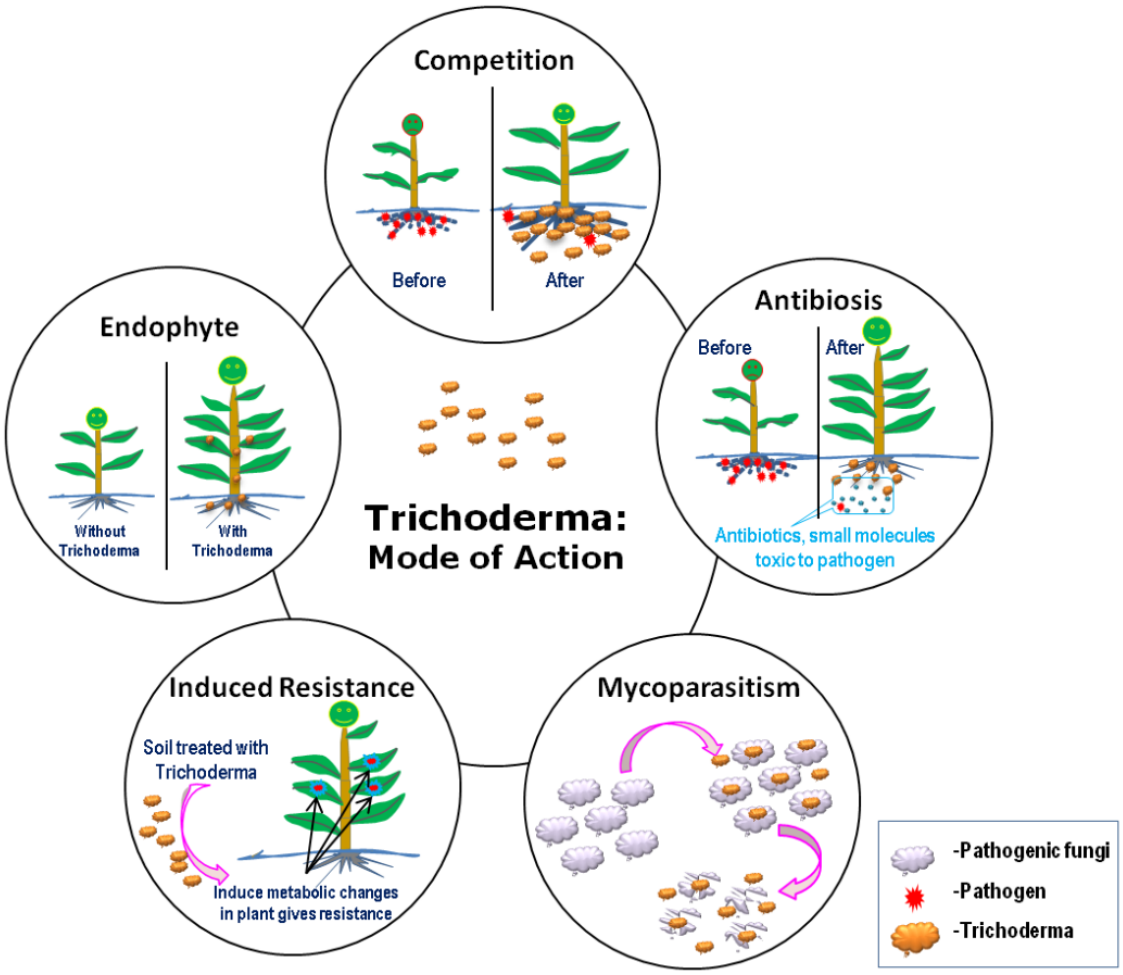

Fig. 3: Model depicting the mode of action of *Trichoderma* spp. against pathogen and their role in plant growth improvement. Credits: Rajesh et al., 2016

These characteristics grant *Trichoderma* a ubiquitous distribution in diverse habitats with significant population densities. The utilization of *Trichoderma* has proven successful in controlling plant pathogens, leading to the development of several commercial products based on different *Trichoderma* species.

Summary of Chapters:

Chapter I: In the first chapter, a new and alarming phenomenon is investigated: the widespread decline of *Fraxinus* species in central-northern Italy. Due to the vastness of this phenomenon and the severity of its impact, it can be considered a true phytosanitary emergence. The research conducted led to the identification for the first time of new etiologic agents affecting these tree species, some of which important, cosmopolitan pathogens. Research conducted with highly sensitive and accurate diagnostic tools (both microbiological and molecular) and wide-ranging epidemiological investigations, carried out for several years, led to ascribe the decline of *Fraxinus* formations to two distinct groups of emerging pathogens: fungi of Botryosphaeriaceae family and oomycetes of the *Phytophthora* genus. The Botryosphaeriaceae family includes 22 genera, the most common being *Botryosphaeria, Diplodia, Dothiorella,* and *Neofusicoccum*. The species identified in this study, cause a wide range of symptoms in *Fraxinus* spp., such as stem and branch cankers, shoot and foliage dieback, and in some cases, death of young regeneration. Stress conditions triggered by global warming have led to epidemic outbreaks by latent Botryosphaeriaceae and emerging pathogens of the genus *Phytophthora*. *Phytophthora* spp. represent in fact another significant group of emerging, lethal pathogens in many global forest areas. Depending of the taxon, *Phytophthora* spp. exhibit a soil-borne or an airborne transmission lifestyle. These oomycetes induce a broad range of often aspecific symptoms in affected hosts; in response to damage to bark and roots, the canopy manifests nonspecific symptoms like yellowing and progressive or sudden decline. The oomycetes described in this study *P. acerina, P. bilorbang, P. cinnamomi, P. hydropathica, P. lacustris, P. multivora, P. plurivora, P. polonica, P. pseudocryptogea, P. pseudosyringae*, and *P. syringae* are reported for the first time on *Fraxinus* spp. in Italy. Based on pathogenicity evidence, it can be assumed they also play a significant role in the decline of *Fraxinus* spp..

Chapter II: In view of the above, and given the ecological importance of *Fraxinus ornus* (common names: "flowering ash" or "manna ash"), especially in central Italy, it was considered useful to deepen the understanding of the decline phenomenon on this particular species. Flowering ash turned out to be highly susceptible to pathogens never investigated before. *B. dothidea* caused extensive and recurring cankers along the stem. Its pathogenicity was confirmed by pathogenicity tests. Symptoms were also evident on branches and shoots of young plants, including shoot folding and foliage dieback by *D. fraxini*. These symptoms are precursors of tree decline, which is also favoured by environmental stresses. The research

demonstrated the pathogenicity ad virulence of these agents and clarified the crucial role that some prominent members have in the decline/dieback of ash formations in investigated areas. **Chapter III:** The most ambitious goal of all diagnostic and epidemiological investigations is the development of effective tools and strategies for the control of pathogens and/or for the mitigation of their impacts. Biological control was one of the main lines along which this research developed. Specifically, attention was paid to an emerging disease of chestnut fruits: brown or chalky rot of nuts. The agent responsible for this new disease is the fungus *Gnomoniopsis castaneae* Tamietti (syn. *Gnomoniopsis smithogilvyi* L. A. Shuttleworth, E. C. Y. Liew, and D. I. Guest) (Shuttleworth et al., 2016). The impact of this disease has reached alarming proportions in the last decades, probably exacerbated also by climate change. In the third chapter, we report efforts undertaken to achieve biological control of *Gnomoniopsis castaneae* in chestnut stands, by exploiting the antagonistic capabilities of fungi of the genus *Trichoderma*. To address this challenge, a biological control method based on tree endotherapy was developed. Over a two-year experimentation period, test chestnut trees were treated with a solution containing competent, selected strains of *Trichoderma* spp.. Throughout the whole study period, the effect of the biocontrol treatments on tree health and the incidence of *G. castaneae*, and other relevant parameters, were monitored. Results proved endotherapic treatment with *Trichoderma* spp. to significantly reduce the incidence of the disease, thus contributing to the control of nut rot. This approach offers a sustainable, eco-friendly alternative to conventional practices. because it allows to reduce dependence on chemical pesticides. On the other hand, in the fruit chestnut grove, considered to all intents and purposes a forest, treatments with synthetic pesticides are not allowed, so biocontrol was the only solution that could be adopted.

Aim of the thesis

The aims of this research were: a) to ascertain the causes of a severe dieback of *Fraxinus* species, with particular emphasis on the die-off of *Fraxinus ornus*, which in central Italy is very widespread, to the point of becoming an alarming phytosanitary issue; b) to investigate some epidemiological aspects of this dieback, by also taking into consideration environmental stress factors that could contribute to the onset and the development of the phenomenon; and c) to develop possible control strategies against an harmful, economically important plant pathogen.

The dieback of *Fraxinus* spp. taken into consideration was a completely new topic, as no one had ever investigated it before. The investigations led to the identification of new aetiological agents affecting these tree species. Some of these taxa are known cosmopolitan pathogens. Two distinct groups of emerging pathogens turned out to be involved in the decline of *Fraxinus*: fungi of the Botryosphaeriaceae family and oomycetes of the genus *Phytophthora*. Some *Phytophthora* species, like some Botryosphaeriaceae, being tolerant taxa, have benefited from climate change, particularly in the Mediterranean environment. These pathogens play an important role in the development of severe decline symptoms, causing considerable product losses and significant economic losses, not only in forestry but also in agriculture and in nursery production.

Another issue was the development of an effective biocontrol method for the management of an emerging as well as alarming new disease of chestnut fruits: brown or chalky nut rot, caused by the ascomycete *G. castaneae*. Given the severity of the disease, which heavily curtails nut production, it has become imperative to develop effective tools for its control and/or mitigation. The protocol involved the use of three species of *Trichoderma* administered via endotherapic treatments. This was a completely new approach as never endotherapy had been used before for the biocontrol of a disease through the use of *Trichoderma* in forests.

These topics are described and discussed in detail in the following three chapters, two of which have already been published in international scientific journals and one of which is still under review.

Chapter I

Pathogenic fungi and oomycetes causing dieback on *Fraxinus* species in the Mediterranean climate change hotspot region

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Pathogenic fungi and oomycetes causing dieback on *Fraxinus* species in the Mediterranean climate change hotspot region

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Environmental changes are occurring on a global scale, but their effects are most pronounced in climate change hotspot zones, such as the Mediterranean basin. Within this area Italy, extending from its southern coasts in the core of the Mediterranean Sea to its northernmost pre-Alpine and Alpine regions, is characterized by a variety of climatic conditions and vegetation types. Surveys conducted in 2018–2022 in forest formations of Central-Northern Italy revealed that the enhanced warming trend and irregular distribution of precipitations are strongly impacting the health of *Fraxinus* species, with some pathogenic fungi and oomycetes being important contributing factors to the decline of the three main ash species growing there: common ash (*Fraxinus excelsior*), flowering ash (*Fraxinus ornus*), and narrow-leaved ash (*Fraxinus angustifolia*). Isolation from symptomatic plant material collected countrywide under different site conditions and pathogenicity tests revealed a complex phytopathological framework, with several pathogenic species in addition to *Hymenoscyphus fraxineus* involved with a prominent role in the ash dieback etiology. Key microbial taxa included the fungal and oomycete pathogens *Botryosphaeria dothidea*, *Diplodia fraxini*, *Diplodia subglobosa*, *Phytophthora acerina*, and *Phytophthora plurivora*. The disease impact was higher on sites where ash trees grew under environmental stress (i.e., areas characterized by mild dry winters, hot summers with intense and prolonged drought) and exhibited reduced vigor, also as a consequence of anthropogenic interference (i.e., silvicultural management and fires). The identified causative agents are emerging pathogens that thrive under warmer conditions, their impact in the investigated areas being prevalent compared to *H. fraxineus*, which appears to be restricted on the Italian peninsula to the cooler and wetter valleys of the Alps and Central-Northern Apennines.

KEYWORDS

ash-tree dieback, stem cankers, leaf and shoot blight, collar necrosis, root diseases, invasive pathogens, climate change

1. Introduction

The Mediterranean basin lies in a transition zone between the semi-arid climate of North Africa and the temperate and rainy conditions of central Europe, affected by interactions between temperate and tropical processes (Giorgi and Lionello, 2008). Due to their particularly favorable geographical and climatic features, Mediterranean regions are characterized by an enormous floral and faunistic diversity, recognized as the second most important biodiversity hotspot on the planet (Myers et al., 2000). The vastness and heterogeneity of this large geographical area allows the survival of over 25000 species, distributed in countless habitats, ranging from the coastal areas, islands and typical low-altitude formations to the closely subalpine and alpine regions; this high biodiversity is mostly linked to intense processes of speciation and extinction during the Quaternary age (Cowling et al., 1996; Myers et al., 2000).

Despite their relative integrity, Mediterranean forests show a high fragility and vulnerability to several natural and human-induced threats such as fires, pests and pathogens, habitat destruction and deforestation (Linaldeddu et al., 2014; Nunes et al., 2022). Climate change has also threatened the survival of these habitats in recent decades; due to its features and geographic position, the Mediterranean basin is considered one of the most prominent climatic hotspots on the planet, representing one of the areas more vulnerable to the impact of climate change in the future (Giorgi, 2006; Giorgi and Lionello, 2008).

Some authors have investigated the profound changes taking place in these regions, correlating them directly to the global climate change; the main factor is a much higher increase in temperatures than the rest of the planet (Ulbrich et al., 2012; Lionello and Scarascia, 2018). In addition, an irregular distribution of the rainfall regime characterizes the Mediterranean area, with increasing and anomalous episodes of drought alternating with extreme events and brief very rainy periods (Valdes-Abellan et al., 2017).

In this scenario of radical change for natural environments, trees are often under conditions of accentuated stress, exposing them more to diseases and pests and posing the potential for forest decline phenomena. These involve a complex group of abiotic and biotic elements and contributors to the losses in tree health and increasing mortality (Manion, 1981; Brasier et al., 1993). During the last three decades, extensive dieback and mortality phenomena have been affecting many European forests, with a greater incidence in the Mediterranean basin (Scanu et al., 2015; Bregant et al., 2020, 2023). One of the most significant examples characterizing the Mediterranean areas with particular incidence is certainly oak decline; recently, many studies have investigated the causes of these widespread phenomena, confirming the direct correlation between climate change as a predisposing factor and pathogens as primary cause of death (Moricca and Ragazzi, 2008; Moricca et al., 2016).

Unlike vast forests involved in decline phenomena, like oak-dominated forests, less widespread but ecologically important *Fraxinus* formations have received relatively little attention. However, in various regions of the Mediterranean in recent years there has been a progressive decline and dieback of the three species of the genus *Fraxinus* that are the main representatives of the genus, namely common ash (*Fraxinus excelsior* L.), flowering ash (*Fraxinus ornus* L.), and narrow-leaved ash (*Fraxinus angustifolia* Vahl.). The damage was particularly serious in some areas, where high mortality, especially in young seedlings, caused a strong limitation to natural regeneration. The attacked trees exhibited a variety of symptoms, the most typical being: sunken cankers on the stem and branches, with a characteristic wedge-shaped necrotic sector in cross section; leaf and shoot blight, resulting in a progressive dieback of the canopy; production of tarry exudates on the lower stem; root and collar rot; in response to the bark and root damages, the canopy evidenced non-specific symptoms of progressive or sudden decline (Orlikowski et al., 2011; Linaldeddu et al., 2020a; Peters et al., 2023).

All these variable symptoms represent a complex syndrome that substantially differ in its etiology and pattern from the simple pathosystem model ash dieback–*Hymenoscyphus fraxineus*. Regarding this latter fungus, it has being expanding since 2009 in various Italian regions starting from the North-Eastern Alps to some areas in the center of the country along the Apennines (Ogris et al., 2010; Luchi et al., 2016; Migliorini et al., 2022). The disease involves all the three above-named ash species, with particular incidence on common ash (*Fraxinus excelsior*) (Panconesi et al., 2014; Rigling et al., 2018). This helotiaceous fungus prefers the cold and humid valleys of the mountain areas of North Italy and North-Central Apennines, its current southern range being some scattered sites in mountain areas with Mediterranean climatic conditions, characterized by cold and snowy winters and cool summers with absence of drought (Migliorini et al., 2022). However, it is unlikely that it will succeed in expanding southward, being limited by the unfavorable conditions of the Mediterranean climate, characterized by mild-dry winters and hot summers with prolonged droughts even in mountainous areas.

This different and more complex phytopathological framework of ash decline in the Mediterranean region emerged in some recent studies and observations in Italy (Benigno et al., 2019; Linaldeddu et al., 2020a) and, paralleled by similar findings in Slovenia, (Linaldeddu et al., 2022), prompted the present investigation, aimed at clarifying the possible role of the new causal agents involved. There is compelling evidence that the rapid changing of climatic conditions occurring in the Mediterranean region is altering the ecology, biogeography and above all infection biology of plant pathogens, creating conditions conducive to new disease emergence and spread (Dukes et al., 2009; Sturrock et al., 2011). These changes markedly alter the relationship between pathogens and their hosts (Sturrock et al., 2011). Some groups of pathogens in particular, like some members of the *Botryosphaeriaceae* family, seem to gain advantage from and thrive under warmer conditions, spreading pervasively over new hosts and areas (Hansen, 2008; Rehfeldt et al., 2009; Venette, 2009). Furthermore, the increasing temperature and altered precipitation regimes also affect the physiology of trees while, at the same time, drought conditions may compromise the fine roots, making trees more susceptible to water stress and attack by root oomycete pathogens (Ginetti et al., 2014; Haavik et al., 2015; Moricca et al., 2016; Colangelo et al., 2018).

In this study, we present new insight into the infection and aggressive colonization of *Fraxinus* species by several emerging pathogens in Central-Northern Italy, with identification of the fungal (endophytic and canker-associated *Botryosphaeriaceae*) and oomycete (*Phytophthora*) species involved, proof of pathogenicity, and elucidation of the key role of some of these pathogens in the dieback of ash species in the investigated areas.

2. Materials and methods

2.1. Sampling and isolation procedures

Investigations were conducted in 40 ash formations distributed from the plains to the mountainous areas in four regions of Central and North-eastern Italy: Toscana, Emilia Romagna, Veneto, and Friuli Venezia Giulia. Survey areas involved the natural ecological range of all three Italian spontaneous ash species: *Fraxinus angustifolia*, *F. excelsior*, and *F. ornus* (**Supplementary Table 1**). Study sites ranged from 0 to 1424 m. a.s.l, covering the entire altitude range for *Fraxinus* spp. in these regions and including the natural reserve of the lowland forest Boscone della Mesola (site 40). Forest sites were characterized by very different meteorological and climate conditions (**Supplementary Table 1**).

From spring 2018 to summer 2022 trees in each site were visually checked for the presence of disease symptoms on canopy (shoot blight, branch canker, bleeding canker), collar and roots (bark necrosis, exudates and root rot). A roughly 50 m long transect was established to evaluate disease incidence and mortality rate, estimated as the number of symptomatic individuals out of the total number of trees (DI = n/N × 100) and the number of dead trees out of the total number of trees (M = d/N × 100), respectively (Moricca et al., 2012a; Linaldeddu et al., 2020a). An amount of 362 samples representative of all symptoms observed on roots including rhizosphere (R = 75 samples), at the collar (TC = 21) and on main stem and branches (C = 262) (**Supplementary Table 1**). The highest number of samples was collected from flowering ash (211), followed by common ash (116 samples) and narrow-leaved ash (35).

All branch, stem and collar samples were taken to the laboratory to be visually examined and the outer bark surface was initially disinfected with 90% ethanol and then removed with a sterile scalpel. Isolations were performed from about 5 mm^2 fragments of inner bark and xylem cut aseptically from the margin of necrotic lesions (Panzavolta et al., 2018; Linaldeddu et al., 2020a). All fragments were placed on 90 mm Petri dishes containing potato dextrose agar (PDA, Oxoid Ltd., UK). After incubation at 25°C for 5–7 days in the dark, hyphal tips from the margin of emerging fungal colonies were sub-cultured onto half-strength PDA and incubated at room temperature under natural daylight to enhance sporulation.

Isolation of root rot agents was performed as reported by Bregant et al. (2020). In the laboratory root and rhizosphere samples were placed in a plastic box and flooded with 2 L of distilled water. After 24 h, young cork oak and elder leaves were placed on the water surface and used as baits. Boxes were kept at 20°C under natural daylight and after 5 days, leaves showing necrotic lesions were cut in small pieces (2–3 mm^2) and placed on 90 mm Petri dishes containing PDA supplemented with 100 ml/L of carrot juice, 0.015 g/L of pimaricin and 0.05 g L^{-1} of hymexazol (PDA +) (Linaldeddu et al., 2020b). Isolation of root rot agents was also directly attempted from roots. Necrotic root tissues were cut in 2 cm long samples, externally disinfected with 90% ethanol, rinsed in distilled water, blotted dry on filter paper and then placed onto PDA + . Petri dishes were incubated in the dark at 20°C and examined every 12 h. Hyphal tips from the emerging colonies were sub-cultured on carrot agar (CA) (Erwin and Ribeiro, 1996) and PDA and incubated at 20°C in the dark. To enhance sporangia production, CA plugs (5 mm diameter) of each isolate were placed in Petri dishes containing unsterile pond water. Sporangial production was assessed every 12 h for 7 days by microscopic observation.

2.2. Isolate identification

Molecular analysis was used to confirm the identification of all isolates at species level. Instagene Matrix (BioRad Laboratories, Hercules, CA, USA) was used to extract genomic DNA from mycelium of 5-day-old colonies grown on PDA and incubated at 20°C in the dark. The universal primers ITS1 and ITS4 were used to amplify the internal transcribed spacer regions (ITS), including the complete 5.8S gene (White et al., 1990). Polymerase chain reaction (PCR) mixtures and amplification conditions were as described by Linaldeddu et al. (2020a). The PCR products were purified using a EUROGOLD gel extraction kit (EuroClone S.p.A., Pero, Italy) following the manufacturer's instructions. ITS regions were sequenced in both directions with the primers used for amplification by the BMR Genomics DNA sequencing service (BMR Genomics, Padua, Italy) and by the CIBIACI University service (Florence, Italy). The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza, Inc., Seattle, WA, USA) and then compared with reference sequences (ex-type culture or representative strains) available in GenBank (NCBI/EMBL) using the BLAST search function (Altschul et al., 2010). Isolates were assigned to a species when their sequences were at least 99.9% homologous to the sequence of type material or representative isolates. ITS sequences from representative isolates obtained in this study were deposited in GenBank (**Table 1**).

For *Botryosphaeriaceae* species ITS sequences of eight representative isolates obtained in this study were compiled in a dataset together with sequences of other 15 isolates belonging to the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, and *Neofusicoccum*. For *Phytophthora* species ITS sequences of 11 representative isolates obtained in this study were compiled in a dataset together with sequences of other 19 isolates belonging to the phylogenetic clades 2, 3, 6, 7, 8, and 9.

Sequences were aligned with ClustalX v. 1.83 (Thompson et al., 1997), using the parameters reported by Bregant et al. (2020). Maximum likelihood (ML) analyses were performed with MEGA-X 10.1.8, including all gaps in the analyses. The best model of DNA sequence evolution was determined automatically by the software (Kumar et al., 2018).

2.3. Pathogenicity tests

The pathogenicity of four *Phytophthora* species, *P. acerina* Ginetti, Jung, Cooke and Moricca, *P. cinnamomi* Rands, *P. plurivora* Jung and Burgess and *P. pseudosyringae* Jung and Delatour, was tested on 3-year-old common ash seedlings grown in plastic pots (10 cm diameter, 1 L volume). The four species of *Phytophthora* were chosen taking into account: (a) their

TABLE 1 Number of isolates obtained from each ash species from rhizosphere (R), collar tissue (TC), and canker (C) samples in the investigated sites.

Species	ITS GenBank code	*Fraxinus angustifolia*			*Fraxinus excelsior*			*Fraxinus ornus*			Sites	Average temperature range(s) (°C)*	Average annual precipitation range(s) (mm)*
		R	TC	C	R	TC	C	R	TC	C			
Armillaria mellea	OR141502	–	–	–	–	3	–	–	–	–	1	13.7	986
Botryosphaeria dothidea	OR141137	–	–	–	–	–	4	–	–	20	1–20, 23, 35	4.0–15.1	887–1387
	OR119871												
Diplodia fraxini	OR140928	–	–	9	–	3	23	–	–	14	1, 5–8, 12, 13, 19, 20, 22–24, 28, 29, 31, 33–35, 37, 40	4.0–15.1	650–2050
	OR177960												
D. mutila	OR140929				–	–	5	–	–	–	21, 24	6.1–6.5	1250–2050
D. seriata	OR140930	–	–	1	–	–	7	–	–	7	2, 7–9, 13, 19, 20, 23, 34, 40	4.0–15.1	650–1387
	OR177959												
D. subglobosa	OR140931	–	–	–	–	–	18	–	–	5	3, 4, 6, 9, 20, 21, 24, 28, 29, 34	6.1–15.0	887–2050
	OR177962												
Dothiorella iberica	OR140932	–	–	–	–	–	–	–	–	1	34	14	1300
Do. symphoricarposicola	OR140933	–	–	–	–	–	1	–	–	–	20	6.5	1250
Hymenoscyphus fraxineus	OR140935	–	–	–	–	–	12	–	–	–	20, 23, 24, 31	4.0–7.6	1250–2050
Neofusicoccum parvum	OR140934	–	–	1	–	–	2	–	-	13	1–6, 8, 28, 37	13–15	887–1585
	OR177966												
Phytophthora acerina	OR140916	–	–	–	12	4	–	1	–	–	20, 22, 24, 28, 31, 33	6.1–13.0	1250–2050

(Continued)

TABLE 1 (Continued)

Species	ITS GenBank code	*Fraxinus angustifolia*			*Fraxinus excelsior*			*Fraxinus ornus*			Sites	Average temperature range(s) (°C)*	Average annual precipitation range(s) (mm)*
		R	TC	C	R	TC	C	R	TC	C			
P. bikorbang	OR140917	1	–	–	–	–	–	–	–	–	40	13	650
P. cinnamomi	OR140918	–	–	–	6	2	–	4	1	–	25, 29, 36, 40	10.5–13.8	650–1460
P. hydropathica	OR140919	3	–	–	–	–	–	–	–	–	40	13	650
P. lacustris	OR140920	12	–	–	–	–	–	–	–	–	40	13	650
P. multivora	OR140921	4	–	–	–	–	–	–	–	–	38,40	13.0–13.6	650–1190
P. plurivora	OR140922	10	1	-	21	5	–	5	–	–	20, 21, 25–30, 32, 34, 36, 38–40	6.5–13.8	650–2390
P. polonica	OR140923	2	–	–	–	–	–	–	–	–	40	13	650
P. pseudocryptogea	OR140924	3	–	–	–	–	–	–	–	–	37, 40	13.0–13.4	650–1132
P. pseudosyringae	OR140925	–	–	–	3	1	–	–	–	–	20, 23	4.0–6.5	1250–1387
P. syringae	OR140926	1	–	–	–	–	–	–	–	–	40	13	650

*Where only one value is reported it is because the microorganism was found at only one site; two values report average extreme values (recorded in sites with extreme values).

high isolation frequency; (b) the severity of symptoms observed in nature; and (c) the absence of information as common ash pathogens. Eight common ash seedlings were inoculated with a representative isolate of each species, and six were used as control. Inoculation point at the collar was surface-disinfected with 70% ethanol and a small piece of outer and inner bark (5 mm diameter) was removed with a flamed cork borer and replaced with an agar-mycelium plug, of the same size, taken from the margin of an actively growing pure culture colony. The inoculation point was covered with cotton wool soaked in sterile water and wrapped in a piece of aluminum foil. Controls were inoculated with a sterile PDA plug applied as described above. All inoculated seedlings were kept in field conditions at 10 to 34°C and watered regularly for 30 days. At the end of the experimental period, seedlings were checked for the presence of disease symptoms, the outer bark was carefully removed with a scalpel and the length of necrotic lesion surrounding each inoculation point was measured. Re-isolation of fungal isolates was attempted by transferring 5 pieces of inner bark taken around the margin of the necrotic lesions onto PDA + . Growing colonies were sub-cultured onto CA and PDA, incubated in the dark at 20°C and identified by morphological and molecular analysis (ITS region).

2.4. Statistical analyses

Pathogenicity assay data were checked for normality, then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined using Fisher's least significant differences (LSD) multiple range test ($P = 0.05$) using XLSTAT software (Addinsoft SARL, New York, NY, USA).

3. Results

3.1. Field survey

Symptoms of ash decline with high mortality were common in some hilly areas of Central Italy on flowering ash and everywhere in North-East Italy on common ash.

In Central Italy, disease severity was high on flowering ash, with typical *Botryosphaeria* cankers and dieback on natural regeneration. Cankers initials appeared as small, sunken, brown-purplish necroses; lesions then extended longitudinally, giving rise to narrow, elongated cankers. Cankers were often multiple along the axis, causing wilting and dieback of young trees. Leaf and shoot blight with crown dieback were also frequently observed (Figure 1).

In North-East Italy, *Fraxinus excelsior* was severely affected, exhibiting a range of aerial symptoms including partial or complete dieback of the crown, abnormal production of epicormic shoots, bark discolorations and sunken cankers. The same symptoms but with a lower incidence were observed on the other two species of ash, in particular on *F. angustifolia*.

Disease incidence was very high, ranging between 70 and 100% among the sites, with a mortality range of 30–70%; the disease symptoms were observed on trees of all ages, with particular virulence and mortality on young seedlings, often reaching 100% of sudden death.

In addition to these common canopy symptoms, the same plants often exhibited root and collar rot, necrosis of inner bark and wood tissues in the basal part of the stem and in some cases bleeding cankers with production of blackish exudates. Root symptoms were often associated with forms of sudden death on young and mature trees. The complex symptomatology was also accentuated by the presence of some secondary pathogens and wood decay fungi, such as *Armillaria* spp. (Figure 1).

3.2. Etiology

Isolation performed on 362 ash samples yielded a total of 251 isolates belonging to 21 different species of oomycetes (102 isolates), ascomycetes (146) and basidiomycetes (3). Of these, 132 isolates were obtained from *Fraxinus excelsior*, 71 from *F. ornus* and 48 from *F. angustifolia* (Table 1). With respect to the type of sample, 143 isolates were obtained from cankers, 83 from roots and rhizosphere and 20 from necrotic inner bark tissues collected at the collar level. Based on morphology, colony appearance and ITS sequence data the 102 isolates of oomycetes were identified as *Phytophthora plurivora* Jung and Burgess (42 isolates), *Phytophthora acerina* Ginetti, Jung, Cooke and Moricca (17), *Phytophthora cinnamomi* Rands (13), *Phytophthora lacustris* Brasier, Cacciola, Nechw., Jung and Bakonyi (13), *Phytophthora multivora* Scott and Jung (4), *Phytophthora pseudosyringae* Jung and Delatour (4), *Phytophthora hydropathica* Hong and Gallegly (3), *Phytophthora pseudocryptogea* Safaief., Mostowf., Hardy and Burgess (3), *Phytophthora polonica* Belbahri, Moralejo and Lefort (2), *Phytophthora bilorbang* Aghighi, Hardy, Scott and Burgess (1) and *Phytophthora syringae* Kleb. (1) (Table 1). The 146 fungal isolates belonged to 9 species of ascomycetes in the families *Botryosphaeriaceae* (134) and *Helotiaceae* (12). In particular, colonies were identified as *Diplodia fraxini* (Fr.) Fr. (49), *Botryosphaeria dothidea* (Moug.) Ces. and De Not. (24), *Diplodia subglobosa* A.J.L. Phillips, Deidda and Linald. (23), *Neofusicoccum parvum* (Pennycook and Samuels) Crous, Slippers and A.J.L. Phillips (16), *Diplodia seriata* De Not. (15), *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz and Hosoya (12), *Diplodia mutila* (Fr.) Mont. (5), *Dothiorella iberica* A.J.L. Phillips, J. Luque and A. Alves (1) and *Dothiorella symphoricarposicola* W.J. Li, Jian K. Liu and K.D. Hyde(1). Finally, 3 isolates from *F. excelsior* were identified as *Armillaria mellea* (Vahl) P. Kumm. (*Physalacriaceae*, *Basidiomycota*).

In the phylogenetic analysis of *Botryosphaeriaceae* species 15 terminal clades were resolved. The isolates obtained in this study clustered in eight well-supported clades (ML bootstrap >90%) together with sequences of ex-type cultures (Supplementary Figure 1). Phylogenetic relationships among the *Phytophthora* isolates were elucidated using ITS sequences. In particular, the 11 isolates included in the phylogenetic analysis were distributed in 11 terminal clades, which belong to formally described species (Supplementary Figure 2).

Phytophthora plurivora and *Diplodia fraxini* were the most commonly detected species. The isolates of these two species were obtained from all three investigated ash species (Figure 2). *Phytophthora plurivora* was isolated from root and collar tissues while *D. fraxini* from branch cankers and necrotic lesions at the

FIGURE 1
Overview of symptoms detected on the ash species monitored in the study: extensive canopy dieback of *Fraxinus excelsior* **(A)**, *F. angustifolia* **(B,C)** and *F. ornus* **(D)**; bleeding canker **(E)**, *Phytophthora* flame necrotic lesions at the collar of *F. angustifolia* **(F)**, *F. excelsior* **(G,H)** and *F. ornus* **(I)**; shoot blight **(J)**, sunken canker **(K)** and inner bark discoloration **(L)** of *F. excelsior* and *F. angustifolia* **(M)**; cross-section of branches showing wedge-shaped necrotic sector **(N)**. Red arrow indicates the white mycelium of *Armillaria mellea* on a necrotic lesion caused by *Phytophthora plurivora*.

collar. Also, *D. seriata* was detected on all host species, albeit less frequently than the other two species.

3.3. Pathogenicity tests

At the end of the experimental trial, all common ash seedlings inoculated with *Phytophthora* spp. displayed dark brown inner bark lesions that spread up and down from the inoculation point (Figure 3). *Phytophthora plurivora* and *P. acerina* were the most aggressive species, causing the longest necrotic lesions (Table 2). Control plants did not show any disease symptoms and exhibited faster growth; only a small light brown discoloration restricted to the inoculation point was observed. All *Phytophthora* isolates were successfully re-isolated from the margin on the necrotic inner bark lesions of all seedlings, thus fulfilling Koch's postulates. No *Phytophthora* isolates or other pathogens were recovered from control seedlings.

4. Discussion

A complex of pathogenic fungi and oomycetes resulted associated with stem and branch cankers, leaf and shoot blights, collar necroses and root rot symptoms on common ash, flowering ash and narrow-leaved ash trees. From a phytopathological perspective, it emerged that ash trees in the investigated forest ecosystems live under significant pathogenic constraints, to which at least two distinct groups of major pathogens concur: prominent members of the *Botryosphaeriaceae* family and aggressive and emerging species of the *Phytophthora* genus.

TABLE 2 Mean lesion length ± standard deviation caused by each *Phytophthora* species inoculated on common ash seedlings.

Species	Isolate	Mean lesion length (cm)*	Re-isolation (%)
P. acerina	CB3	3.9 ± 1.1a	100
P. cinnamomi	CB121	2.8 ± 1b	100
P. plurivora	CB122	4.4 ± 1.7a	100
P. pseudosyringae	CB183	2.4 ± 0.4b	100
Control	–	0.5 ± 0.2c	–
LSD critical value		2.03	

*Values with the same letter do not differ significantly at $p = 0.05$, according to LSD multiple range test.

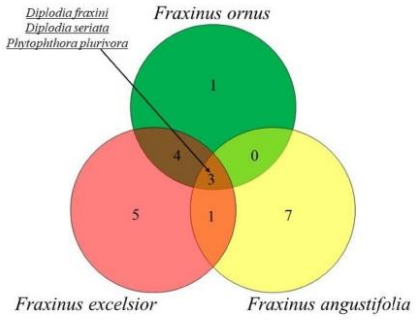

FIGURE 2
Venn diagrams illustrating the number of unique and shared species among ash species.

Other opportunistic pathogens such as *Armillaria* spp., were often found coinfecting declining/dying ash trees. *Hymenoscyphus fraxineus* was found on *F. excelsior* at a few sites but its impact on infected trees appeared less than that caused by the other pathogens. Some species prevailed at one site or another, depending on the particular context and microclimatic conditions. *H. fraxineus* was isolated in the coldest sites (mean annual temperature ranging from 4 to 7.6°C) and with an average annual precipitation ranging from 1250 to 2050 mm.

The *Botryosphaeriaceae* (*Botryosphaeriales*, Ascomycetes) are an emerging family of plant pathogenic fungi affecting various tree and shrub species typical of the Mediterranean area (Ragazzi et al., 1997; Moricca et al., 2008, 2010; Linaldeddu et al., 2016; Moricca and Linaldeddu, 2017). This family encompasses 22 genera, of which *Botryosphaeria*, *Diplodia*, *Dothiorella*, and *Neofusicoccum* are the most common in forest ecosystems (Moricca et al., 2012b; Phillips et al., 2013; Batista et al., 2021). Affected plants can show a wide range of symptoms, the most typical of which are cankers on the stem and branches with a characteristic wedge-shaped necrotic sector in cross section resulting in a progressive dieback of the canopy (Ragazzi et al., 1999a; Linaldeddu et al., 2016; Manca et al., 2020).

The relevance of *Botryosphaeriaceae* as pathogens stands out by taking into consideration the number of disease reports caused by members of this family, that has undergone an exponential increase worldwide, rising from around 100 in the period 1960–2000 to over 1500 in the last 20 years (Scopus, June 2023); this is mainly due to a greater scientific interest in these diseases, the development of new molecular and bioinformatic diagnostic techniques, enabling more accurate identification of fungal taxa, and the ongoing climate change (Batista et al., 2021). The stressful conditions for host species triggered by global warming have provoked the manifestation of epidemic diseases by endophytic and latent species of *Botryosphaeriaceae* (Ragazzi et al., 1999b,c); therefore, the introduction and establishment of invasive *Botryosphaeriaceae* in new areas of the planet driven by the rising temperatures, caused the occurrence of new emerging diseases incited by these fungi in regions previously considered unsuitable to many of them (Batista et al., 2020, 2021).

Diseases caused by *Botryosphaeriaceae* are drawing the attention of researchers especially in the Mediterranean region, consider one of the most striking examples regarding the emerging diseases (Ragazzi et al., 1996; Linaldeddu et al., 2017; Panzavolta et al., 2017; Smahi et al., 2017). Italy is certainly one of the most affected countries within the Mediterranean region. Indeed, countless studies have reported about 60 species of *Botryosphaeriaceae* in this country, resulting in over 250 host-pathogen interactions threatening diverse sectors of primary production (Moral et al., 2010; Urbez-Torres, 2011; Batista et al., 2021; Fiorenza et al., 2023). Although the economic impact is higher in crop production, the diseases caused by *Botryosphaeriaceae* species can also be devastating in forestry, causing losses of biodiversity and impairing ecosystem integrity (Slippers and Wingfield, 2007; Marsberg et al., 2017).

Prominent examples are the decline of Mediterranean vegetation caused by several *Diplodia* and *Neofusicoccum* species (Moricca et al., 2012b), pine shoot blight due to the invasive species *Diplodia sapinea*, oak canker disease due to *D. corticola* and ash dieback caused by *D. fraxini* (Luchi et al., 2014; Cimmino et al., 2016; Manca et al., 2020).

The dieback of ash formations has been associated for a long time, especially in Eastern Europe, to the ascomycete fungus *H. fraxineus* (Kowalski, 2006). Recent investigations in Germany, Italy and Slovenia, as well as this study, better clarify the key role of *Botryosphaeriaceae* species in the etiology of ash dieback (Linaldeddu et al., 2020a, 2022; Peters et al., 2023). In this study, *B. dothidea* was isolated at very high frequency from cankered tree tissues of *F. ornus* individuals in hilly areas of Central Italy. This cosmopolitan pathogen has been isolated worldwide from sites characterized by very different climatic conditions (mean annual temperature between 4 and 15.1°C) and mean annual rainfall between 887 and 1387 mm (Marsberg et al., 2017; Xue et al., 2021). In central Italy, the impact of the disease was greater on poor soils rich in gravel, on slopes facing south or south-west, at sites exposed to high daily temperatures and heat waves during the growing season and generally in stands suffering from drought stress. Mortality was high on young seedlings, especially following long dry periods. *Diplodia fraxini* was the most constantly isolated species from symptomatic ash trees and its virulence was confirmed by independent pathogenicity assays (Elena et al., 2018; Linaldeddu et al., 2020a). This fungus seems to manifest a

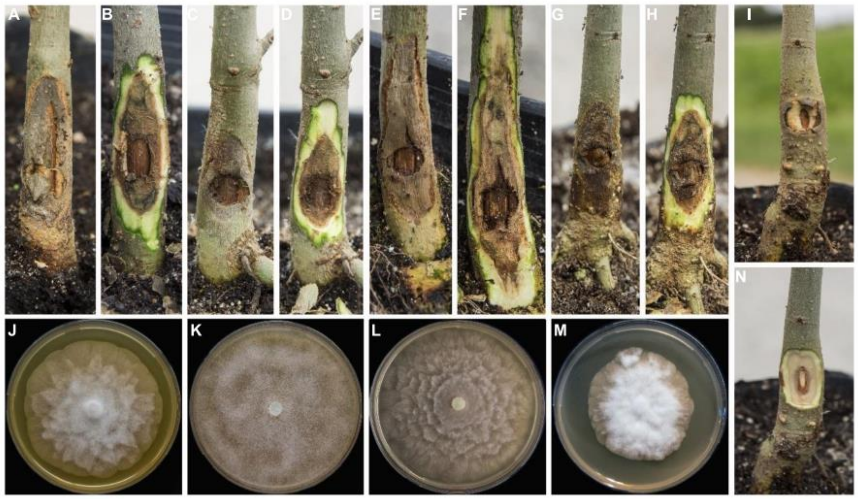

FIGURE 3
Symptoms caused by *Phytophthora acerina* **(A,B)**, *P. cinnamomi* **(C,D)**, *P. plurivora* **(E,F)** and *P. pseudosyringae* **(G,H)** on common ash seedlings. Control seedling **(I,N)**. Colony morphology of *P. acerina* **(J)**, *P. cinnamomi* **(K)**, *P. plurivora* **(L)**, and *P. pseudosyringae* **(M)** on carrot agar after 7 days at 20°C in the dark.

particular host-specificity for the genus *Fraxinus*, including the capacity to produce selective phytotoxic secondary metabolites (Cimmino et al., 2017).

Diplodia fraxini, included for a long time in the *Diplodia mutila* complex, was recently re-instated; therefore, many isolates from *Fraxinus* that were assigned in GenBank to *Diplodia mutila* belong in reality to *D. fraxini*, a fact that demonstrates a wider distribution of this species in Europe (Alves et al., 2014; Linaldeddu et al., 2020a). The current distribution of *D. fraxini* is still unknown; however, the impact of ash dieback, that appear to be growing in central Europe and new areas of the Mediterranean region such as Central Italy, the Balkans and Iberian Peninsula, underline the strong adaptation of this fungus to changing environmental conditions (Alves et al., 2014; Elena et al., 2018; Peters et al., 2023). The plasticity of this pathogen is confirmed by its discovery in numerous sites with very different climatic conditions: average temperature ranging from 4 to 15.1°C and average annual precipitation ranging from 650 to 2050 mm.

Phytophthora spp. are another important group of lethal pathogens that are overbearingly emerging in many forest areas globally (Jung et al., 2016). Most *Phytophthora* species have a soilborne lifestyle, causing primarily root and collar rot on thousands of plant species, but some of them are endowed with an aerial dispersal lifestyle. These oomycetes cause a broad range of symptoms on affected hosts; in response to the bark and root damages, the canopy evidences non-specific symptoms of progressive or sudden decline (Bregant et al., 2020).

Over the past two decades, countless studies have demonstrated the involvement of a large number of soilborne *Phytophthora* species in the widespread declines of forest ecosystems dominated by the *Fagaceae* and the *Betulaceae* in Europe, especially in the Mediterranean basin (Brasier et al., 1993; Jung et al., 2000; Vettraino et al., 2005; Linaldeddu et al., 2014; Scanu et al., 2015; Corcobado et al., 2020; Riolo et al., 2020; Bregant et al., 2023).

In some cases, attacks were a matter of re-emergence of old-known *Phytophthora* species which, after decades of infrequent, sporadic outbreaks, in the past 20 years have come back to cause major epidemic outbreaks. A key to understanding the resurgence of diseases caused by old-known *Phytophthora* related diseases (e.g., the "ink disease" on chestnut) is the current climate trend. It must be considered that the Mediterranean-type climate ecosystems present today conditions particularly conducive to the lifestyle and development of this group of pathogens: the relatively warm and wet winter period can induce an easy zoospore production for host infection in combination with the subsequent long and dry summers that cause severe stress in tree populations (Serrano et al., 2022). In this scenario, an increasing number of *Phytophthora* species are emerging or re-emerging from the Mediterranean areas of the planet, such as SW Australia, California, South Africa and the classic Mediterranean Basin (Burgess et al., 2017).

Crown and root diseases related to *Fraxinus* spp. dieback are to date still little studied. Current knowledge about the association between *Phytophthora* spp. and ash is limited. Until a few years ago, the *Fraxinus* genus was considered resistant to *Phytophthora* attacks, as also ascertained by some pathogenicity tests (Jung and Nechwatal, 2008; Mrázková et al., 2013). The first study to report attacks of *Phytophthora* spp. on natural *Fraxinus* formations took place in 2011 in Poland and Denmark, highlighting root and collar root symptoms, although the *Phytophthora* presence had been ascertained a few years earlier in areas of Central and Eastern Europe such as Germany and the Czech Republic (Mrázková et al., 2010, 2013; Orlikowski et al., 2011; Langer, 2017).

A study conducted by Tkaczyk et al. (2016) found five *Phytophthora* species from common ash in a nature reserve in Poland. In this study, *P. cactorum* and *P. plurivora* appeared to be the most frequent and virulent species associated with common ash (Orlikowski et al., 2011; Tkaczyk et al., 2016). Recent studies have also demonstrated a high susceptibility to *Phytophthora* species of natural riparian formations of *Fraxinus angustifolia* in Sicily and Türkiye (Akilli et al., 2013; Jung et al., 2019). Some studies conducted in Northern and Eastern Europe highlighted a possible synergistic action between canopy and root pathogens (Orlikowski et al., 2011; Tkaczyk et al., 2016; Milenković et al., 2017, 2018; Peters et al., 2023). Other studies have shown a frequent association of declining ash trees with secondary root pathogens such as *Armillaria* spp. (Lygis et al., 2005; Skovsgaard et al., 2010; Langer, 2017; Kranjec Orlović et al., 2020; Peters et al., 2023).

Given the above, this study stands as the most complete census of *Phytophthora* species attacking members of the genus *Fraxinus*, with 11 different species, namely *P. acerina*, *P. bilorbang*, *P. cinnamomi*, *P. hydropathica*, *P. lacustris*, *P. multivora*, *P. plurivora*, *P. polonica*, *P. pseudocryptogea*, *P. pseudosyringae*, and *P. syringae* that are reported for the first time on these hosts in Italy.

The 4 *Phytophthora* species used in the pathogenicity tests, *P. acerina*, *P. cinnamomi*, *P. plurivora*, and *P. pseudosyringae*, selected on the basis of their high isolation frequencies and the severity of symptoms caused on ash trees, confirmed to be aggressive pathogens on ash and provided important information about their impact and pervasiveness in forest ecosystems. In particular, *Phytophthora plurivora* and *P. acerina* proved to be the most virulent species in the artificial inoculation tests, producing the longest lesions on inoculated seedlings. It is noteworthy that both species are members of the former *P. citricola* complex (Ginetti et al., 2014).

Phytophthora plurivora is a root and stem pathogen very widespread in Europe, known to occur in different settings and environments (plantations, nurseries, ornamental green, streams, primary forests) and to cause widespread declines on alder (*Alnus* spp.), beech (*Fagus sylvatica*), and oak (*Quercus* spp.) ecosystems (Jung, 2009; Jung and Burgess, 2009; Lilja et al., 2011; Prospero et al., 2013; Schoebel et al., 2014; Bregant et al., 2023). Having been found at very high frequencies even in ash formations confirms its widespread presence in forest ecosystems and suggests it might have a major role also in the decline of ash formations.

Phytophthora acerina was first reported and described about 10 years ago from a wooded area in a peri-urban park on the Lombardy plain (Northern Italy). Here, it infected and killed thousands of individuals of *Acer pseudoplatanus*, making this tree species literally disappear from the area (Ginetti et al., 2014). The pathogen was able to spread pervasively among water sources, the whole area being rich in water (some plots were in the past cultivated with rice). *Phytophthora acerina* was in fact recovered from streams, ponds, canals, reservoirs, runoffs, irrigation waters, as well as—of course—from bleeding cankers on the stem, root tissues and soil samples taken under the crown of dead/dying trees. The pathogen was reported a few years later, in 2018, causing sudden death to olive trees in the nearby Veneto region, a few hundred kms from the site of its first report. On olive trees *P. acerina* exhibited high virulence as well, causing a range of symptoms such as partial or complete crown dieback, reddening of drying foliage, loss of rootlets and collar rot (Linaldeddu et al., 2020c). These literature data and the high virulence displayed by *P. acerina* on inoculated seedlings as well as on ash trees in the woods prove it to be an extremely dangerous and highly pervasive pathogen, whose host and geographic range is still largely unexplored, but which certainly includes the *Fraxinus* species.

Phytophthora cinnamomi was isolated from ash trees at many sites and this confirms this generalist pathogen to be rather ubiquitous and to become invasive in some areas, often causing enormous damage to natural ecosystems (Hardham and Blackman, 2018). This oomycete shows a rather accentuated seasonality and is favored, in particular, by frequent rains in the spring and by mild winters, a condition typical of the Mediterranean climate; current climate anomalies could significantly modify these conditions, further favoring the epidemiological spread of this pathogen (Serrano et al., 2022). The recent description in the Mediterranean of two species close to *P. cinnamomi* with a further adaptation to even higher temperatures is proof of the vulnerability of natural ecosystems to this group of pathogens in the era of climate change (Scanu et al., 2014; Yang et al., 2017; Bregant et al., 2021).

5. Conclusion

Global climate change is altering site factors, biochemical processes and biotic interactions in natural plant communities (Sturrock et al., 2011). The impact of climate change is exacerbated in climate change hotspots. Italy, a peninsula jutting out into the center of the Mediterranean climate change hotspot region, is experiencing unprecedented mild dry winters, hot summers and prolonged droughts that are seriously impacting forest vegetation and changing the landscape. As the scientific community debates if species migration will be able to keep pace with climate change, with projections about climate-driven range shifts in tree species, forest pathologists tirelessly record extended forest diebacks, invasions by new pathogens and altered densities and distribution of forest tree species within natural plant communities (Singh et al., 2023). That's what seems to be going on with ash formations: common ash, flowering ash and narrow-leaved ash stands are under unprecedented attack by new pathogens and the almost total loss of natural regeneration observed at some locations indicates these species might be retreating from the less favorable sites.

Another reason for concern arises from knowledge on the contrasting infection biologies and lifestyles of the two groups of causative agents involved. The fungal and oomycete pathogens reported here, being endowed with different thermo-hygrometric requirements, could be vicariant throughout the year in their pathogenetic action, with *Phytophthora* spp. being more active in mild and moist seasons and *Botryosphaeriaceae* that become more aggressive during hot and dry periods. If this pattern of vicariance, already observed in oak forests (Moricca et al., 2012c), would also be reproduced in ash stands, it could catch infected ash trees in a deadly spiral, as the two groups of pathogens would act synergistically in weakening ash trees and lead to their death.

The large number of pathogenic species found in this study agrees with the results of a recent research conducted in Germany (Peters et al., 2023), and underlines that while the litter species *H. fraxineus* has been getting the most attention in the last decades,

the most common species associated with *Fraxinus* spp. with ash dieback symptoms are *Diplodia fraxini* and *P. plurivora*. The overall framework indicates that multi-trophic interactions are common in ash stands, representing an important and matter-of-fact aspect of tree-pathogen relationships, and this provides a more realistic picture of what's going on in forests (Pillay et al., 2013; Smahi et al., 2017). Clarifying this complex etiology is critical to assess if, and to what extent, ash disease management and prevention measures can be effectively applied.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary material**.

Author contributions

AB, CB, SM, and BL contributed to conception and design of the study. AB and CB conducted the field and laboratory trials, analyzed the data, and wrote the original manuscript. CA, GR, and BT helped with laboratory investigations, data curation and preparation of the first draft of the manuscript. SM and BL supervised and funding acquisition. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2023.1253022/full#supplementary-material

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Supplementary Material

Pathogenic fungi and oomycetes causing dieback on *Fraxinus* species in the Mediterranean climate change hotspot region

Alessandra Benigno[1*], Carlo Bregant[2], Chiara Aglietti[1], Giovanni Rossetto[2], Beatrice Tolio[2,3,4], Salvatore Moricca[1*], Benedetto T. Linaldeddu[2]

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Supplementary Table 1 - Study sites information and number of root and rhizosphere (R), stem collar (TC) and branch canker (C) samples collected for each *Fraxinus* species.

Sites	Mean annual temperature (°C)	Average annual precipitation (mm)	Altitude (m a.s.l.)	Geographic coordinates		Number of samples		
				Latitude (X)	Longitude (Y)	*F. angustifolia*	*F. excelsior*	*F. ornus*
1	13.7	986	357	43.080174	11.134758	-	-	C(9)
2	13.7	986	341	43.080912	11.133571	-	-	C(12)
3	13.7	986	322	43.081214	11.132678	-	-	C(7)
4	13.7	986	321	43.082047	11.132164	-	-	C(11)
5	13.7	986	321	43.082055	11.130948	-	-	C(11)
6	13.7	986	360	43.082710	11.124901	-	-	C(13)
7	13.7	986	378	43.083941	11.120993	-	-	C(7)
8	15	887	496	43.081768	11.103309	-	-	C(11)
9	15	887	359	43.074419	11.102301	-	-	C(6)
10	15	887	382	43.070660	11.101050	-	-	C(10)
11	15	887	437	43.065790	11.093702	-	-	C(13)
12	14.3	1198	381	43.064221	11.085139	-	-	C(5)
13	14.3	1198	401	43.063078	11.084503	-	-	C(9)
14	14.3	11.98	408	43.063022	11.084015	-	-	C(11)
15	14.3	1198	392	43.062143	11.083937	-	-	C(14)

16	14.3	1198	390	43.061097	11.083421	-	-	C(10)
17	14.3	11.98	449	43.055967	11.082859	-	-	C(12)
18	15.1	995	458	43.061936	11.074833	-	-	C(6)
19	15.1	995	515	43.065178	11.070384	-	-	C(8)
20	6.5	1250	1012	46.471167	12.461170	-	R(10), TC(6), C(21)	-
21	6.5	1250	1215	46.4675090	12.4833650	-	R (5), TC(3), C(6)	-
22	6.5	1250	1060	46.4729600	12.4668290	-	R(2), TC(2), C(2)	-
23	4.0	1387	1424	46.4926405	12.5622738	-	R(2), C(6)	-
24	6.1	2050	1074	46.0994836	12.4160491	-	R(2), TC(2), C(10)	-
25	10.5	1460	478	46.0800680	12.2407450	-	R(3)	-
26	6.9	1375	1008	45.8648319	11.5232058	-	R(5), TC(1)	-
27	10.0	2390	640	46.2817502	12.4523223	-	R(2)	-
28	13.0	1585	437	46.1758573	13.6339655	-	R(3), TC(2), C(5)	-
29	13.8	1426	70	45.9536413	13.5321619	-	R(5), TC(2), C(2)	-
30	7.9	1537	740	46.4997485	13.6281560	-	R(2)	-
31	7.6	1520	1010	46.5379836	13.0856086	-	R(2), CT(1), C(2)	-
32	13.8	1426	100	45.945762	13.562933	-	-	R(3), TC(1)
33	12.0	1650	510	45.986783	13.631923	-	-	R(1), C(2)

34	14.0	1300	23	45.833590	13.560242	-	-	R(1), C(3)
35	13.5	948	50	45.264579	11.717262	-	-	C(5)
36	13.5	948	350	45.318134	11.708450	-	-	R(2)
37	13.4	1132	3	45.788514	13.115748	R(3), C(6)	-	-
38	13.6	1190	2	45.790750	13.188088	R(2), TC(1)	-	-
39	13.7	821	11	45.378336	11.970178	R(1)	-	-
40	13.0	650	0	44.851910	12.252461	R(16), C(6)	-	R(3), C(1)

Supplementary Material

Pathogenic fungi and oomycetes causing dieback on *Fraxinus* species in the Mediterranean climate change hotspot region

Alessandra Benigno[1*], Carlo Bregant[2], Chiara Aglietti[1], Giovanni Rossetto[2], Beatrice Tolio[2,3,4], Salvatore Moricca[1*], Benedetto T. Linaldeddu[2]

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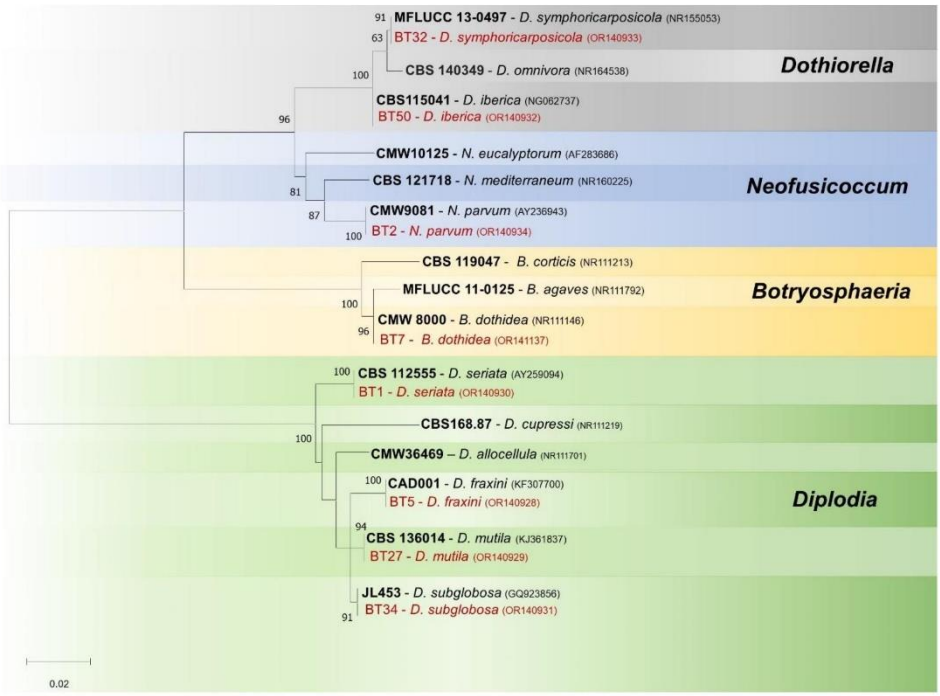
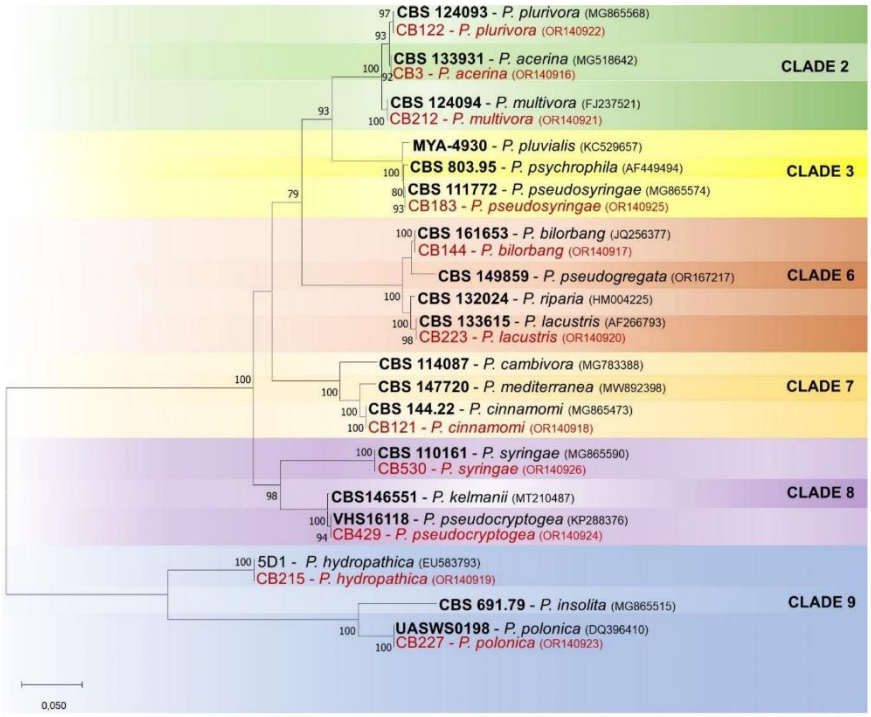
Supplementary Figures



Supplementary Figure 1. Maximum likelihood tree obtained from ITS sequences of *Botryosphaeriaceae* species belonging to the genera *Botryosphaeria, Diplodia, Dothiorella* and

Neofusicoccum. Data are based on the General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap support values in percentage (1000 replicates) are given at the nodes. Ex-type cultures are in bold, and isolates obtained in this study in red. GenBank access codes are in brackets.



Supplementary Figure 2. Maximum likelihood tree obtained from ITS sequences of *Phytophthora* species belonging to 6 out of the 12 major phylogenetic clades (Yang *et al*., 2017). Data are based on the General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap support values in percentage (1000 replicates) are given at the nodes. Ex-type cultures are in bold, and isolates obtained in this study in red. GenBank access codes are in brackets.

Chapter II

Botryosphaeriaceae species associated with stem canker, shoot blight and dieback of *Fraxinus ornus* in Italy

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Article

Botryosphaeriaceae Species Associated with Stem Canker, Shoot Blight and Dieback of *Fraxinus ornus* in Italy

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Abstract: A severe dieback of flowering ash (*Fraxinus ornus* L.) has been observed in north-central Italy in the last decades. Symptoms include typical sunken, light-brown cankers on the stem and branches; vascular discoloration; tip and shoot dieback; and foliage necroses. The disease was more evident at the beginning of the growing season, and more severe on young regeneration. Six *Botryosphaeriaceae* species were consistently isolated from symptomatic plant tissues: *Botryosphaeria dothidea*, *Diplodia fraxini*, *Diplodia subglobosa*, *Dothiorella iberica*, *Dothiorella omnivora* and *Neofusicoccum parvum*. *B. dothidea* and *D. fraxini* expressed higher aggressiveness and showed a widespread incidence, being the species most frequently associated with cankers; the other four species were less virulent and more erratic, occurring mainly on succulent branch tips and foliage. Isolates were characterized using morphological and molecular approaches (colony/conidial phenotyping and rDNA-ITS genotyping). Phylogenetic analysis provided congruent phylogenies depicting the relationships of the six taxa with the most closely related conspecifics. Pathogenicity tests on 2-year-old seedlings confirmed the higher virulence of *B. dothidea* and *D. fraxini*. Extensive, multi-year field surveys at different sites supported the hypothesis that climatic vagaries, mainly heat, water and drought stresses, impaired tree health and vigor, facilitating infection and pervasive colonization by these *Botryosphaeriaceae* species. Environmental stressors are thus the key factor bringing the six fungal pathogens together in a multitrophic interaction with *F. ornus* in a novel, lethal fashion.

Keywords: flowering ash-dieback; cankers; twig blights; leaf necroses; endophytic *Botryosphaeriaceae*; pathogenicity assays; climate change; environmental stress; Mediterranean area

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1. Introduction

Flowering ash (*Fraxinus ornus* L.), also known as "manna ash", is a small- to medium-sized deciduous tree species native to hilly and mountainous mixed forests of southern Europe and southwestern Asia [1]. This tree has an asymmetrical, hemispherical or flattened crown composed of leaves that are 20–30 cm long and odd-pinnate, arranged in 5–9 leaflets that are obovate and 7–10 cm long. The bark is dark grey and smooth, even in adult trees [1]. Though the timber of this species usually has low economic interest, *F. ornus* stands have been maintained in various parts of Europe as coppices for producing firewood and small tool handles [2]. The scarce appreciation of *F. ornus* timber is mainly due to this species' pioneer habit that facilitates its growth on slopes and remote places, favoring the development of small and poorly shaped trunks with many timber defects. On the contrary, these features give *F. ornus* high ecological importance, which is substantiated in its use as a primary component of protective forests and for afforestation of degraded sites [3]. In addition, this species assumes economic value in southern Italy where it is planted to

produce the manna, a bitter-sweet tasting sap that is exuded by the plant, crystallizing in the air into yellow masses, used as a sweetener, laxative, and digestive aid [1]. Due to the poor quality of its wood, *F. ornus* always received little attention from humans, and this is why no serious threat had been documented for this tree species until the reporting in Italy of the ash dieback disease caused by the ascomycete fungus *Hymenoscyphus fraxineus* [4]. *H. fraxineus* occurrence has been documented in some Italian regions since 2009, but although the fungus is able to infect all the ash species growing there, i.e., common ash (*Fraxinus excelsior*), flowering ash (*Fraxinus ornus*) and narrow-leaved ash (*Fraxinus angustifolia*), serious damage has been reported only on *F. excelsior* [5–7].

A diverse and more complex disease framework has been observed in recent years in *Fraxinus* forests, mostly concentrated in Mediterranean-climate areas characterized by lack of rainfall and prolonged drought. The varying set of symptoms observed on *F. ornus* collectively generates an undescribed syndrome, which is quite dissimilar from the well-defined symptomology caused by *H. fraxineus* [8–10]. Indeed, this new syndrome includes sunken cankers on the stem and branches, sometimes appearing numerous along the stem of young plants; a characteristic wedge-shaped necrotic sector in the cross section of declining branches and stems; leaf and shoot blight; and progressive dieback of the canopy [11,12].

The above-described progressive and extensive dieback, which is severely affecting *F. ornus* stands in some areas of central and northern Italy, has increased over the years, leading to the necessity to ascertain the etiologic agent(s) as well as the possible predisposing factors involved [13,14]. Among the predisposing factors that could have activated the onset of this new decline/dieback syndrome, climate change seems to have a central role. Climate warming, in fact, by altering temperature and precipitation regimes at a regional scale, can strongly impact plant growth and physiology on the one hand, and modify the life-history strategies and behavior of associated microorganisms on the other. Regional climate anomalies can thus cause profound changes in natural environments, reducing the resilience of forest formations, increasing tree vulnerability and triggering the onset of new diseases [15,16]. The general climate warming trend is particularly exacerbated in the Mediterranean basin, considered one of areas of the planet most threatened by it. In fact, the Mediterranean zone is expected to undergo a higher increase in temperatures than the rest of the planet combined with prolonged drought events followed by extreme weather conditions [17–20]. In this alarming scenario, the ecology, biogeography and infection strategies of plant pathogens can be altered, creating conditions conducive to new disease emergence and spread [15,16,21].

Among the pathogens that can take advantage of climate change are some prominent members of the *Botryosphaeriaceae*. The *Botryosphaeriaceae* family includes more than 200 species that can affect thousands of plant species worldwide [22]. Many of these fungi are acquiring importance as emerging pathogens due to frequent reports of their relationship with dieback syndromes in forest and agro-forest ecosystems [23]. The endophytic lifestyle of these fungi has long been demonstrated, and the main factors linked to the development of diseases caused by these fungi have been identified in stressful environmental conditions [24]. Among these, physiological plant dysfunctions related to high temperatures and drought have been identified as the main causes of tree impairment and the emergence of *Botryosphaeriaceae*-related diseases [25,26]. Hence, climate change can be considered one of the major drivers of *Botryosphaeriaceae*-related attacks, and this has attracted the attention of researchers, especially in the Mediterranean region [27,28].

Some members of this family have been found in association with *Fraxinus* dieback and potentially involved in the etiology of the decline of ash formations [12,29–31]. In this study, we have investigated the causes of a widespread decline/dieback of *F. ornus* stands in central and northern Italy (Tuscany, Veneto and Friuli Venezia Giulia), with identification of the fungal species involved, proof of pathogenicity and elucidation of the key role of some endophytic and canker-associated *Botryosphaeriaceae* in the development of the observed decline/dieback syndrome.

2. Materials and Methods

2.1. Field Surveys and Sampling

Field surveys were conducted in twenty-five *F. ornus* stands mixed with other broadleaved tree species, ranging in altitude from 50 to 722 m a.s.l., distributed in three Italian regions: Tuscany, Veneto and Friuli Venezia Giulia. Symptoms affecting *F. ornus* individuals were registered for a six-year period from 2018 to 2023 in each analyzed area and geographical coordinates were recorded for each stand (Table 1). In order to identify the causal agents, samples were collected from symptomatic shoots, branches and stems. Portions of stems with cankers were retrieved by dissecting the stems of 91 collected ash trees into 20 cm long fragments. Each collected sample was placed in a bag, classified by location and taken to the Plant Pathology Laboratory, DAGRI (University of Florence; Italy) for laboratory analyses.

Table 1. Sites surveyed for *Fraxinus ornus* dieback and number of collected samples.

Sites	Region	Altitude (m. a.s.l.)	Geographic Coordinates		Number of Samples Collected
			Latitude (X)	Longitude (Y)	
1	Tuscany	498	43.065824°	11.070266°	3
2	Tuscany	448	43.055333°	11.081245°	6
3	Tuscany	439	43.050120°	11.082984°	4
4	Tuscany	722	43.033821°	11.094413°	3
5	Tuscany	554	43.024153°	11.101669°	1
6	Tuscany	553	43.023907°	11.101867°	3
7	Tuscany	545	43.021426°	11.093438°	3
8	Tuscany	312	43.014474°	11.091555°	3
9	Tuscany	306	43.002083°	11.111248°	4
10	Tuscany	323	43.091973°	11.170991°	4
11	Tuscany	334	43.090548°	11.171011°	4
12	Tuscany	347	43.142534°	11.115229°	5
13	Tuscany	333	43.140372°	11.112651°	5
14	Tuscany	431	43.172679°	11.045179°	2
15	Tuscany	438	43.165697°	11.032799°	2
16	Tuscany	609	43.153484°	11.031911°	4
17	Tuscany	556	43.154064°	11.035591°	4
18	Veneto	240	45.309950°	11.773347°	6
19	Veneto	216	45.289807°	11.703996°	5
20	Veneto	184	45.300556°	11.767728°	4
21	Veneto	259	45.318134°	11.708450°	2
22	Veneto	50	45.264882°	11.717811°	5
23	Friuli Venezia Giulia	567	45.988357°	13.634403°	3
24	Friuli Venezia Giulia	64	45.945518°	13.552939°	4
25	Friuli Venezia Giulia	187	45.8334681°	13.580679°	2

2.2. Fungal Isolations

Samples were first examined under a Leica Wild M8 stereoscope (Leica Microsystems, Heerbrugg, Switzerland) to assess the possible presence of pycnidia or ascomata. Fungal isolations were then conducted. Samples were surface-disinfected by washing with 3% sodium hypochlorite solution (NaOCl) for 1 min and rinsing in sterile distilled water. After disinfection, plant tissues were dried on sterile absorbent paper. Outer bark was removed by using a sterile scalpel. Five fragments (4–5 mm^2) of inner bark and xylem tissues were then cut aseptically from the margin of the healthy and necrotic parts. Isolations were performed on 9 cm diameter plastic Petri dishes filled with 2% Potato Dextrose Agar (Liofilchem srl, Roseto degli Abruzzi, Teramo, Italy) amended with 250 mg L^{-1} ampicillin

+ 10 mg L^{-1} rifampicin. All Petri dishes were incubated in the dark at 24 ± 1 °C for 7 days. Subsequently, grown colonies were subcultured onto new 2% PDA Petri dishes.

2.3. Morphological Identification

Fungal cultures were initially grouped into morphotypes based on colony growth and phenotypic characteristics, including surface and reverse colony appearance observed after 10 days of incubation on PDA at 25 ± 1 °C in the dark, and morpho-biometric data of conidia. To induce the production of fruiting bodies and conidia by each fungal taxon, each isolated morphotype was transferred onto water-agar supplemented with autoclaved pine needles [32] and incubated at room temperature under UV light. After 14 days of incubation, each isolate was inspected under a Wild M8 stereoscope for the production of pycnidia. Using a sterile scalpel, pycnidia were collected and mounted on glass slides in 100% lactic acid and observed under a Zeiss light microscope (ZEISS, Jena, Germany). Morphology of conidia was determined at ×40 magnification by measuring 100 conidia from each morphotype (length × width). Based on morphological identification, the isolation frequency of *Botryosphaeriaceae* was calculated using the following formula: F = (NBot/NTot) × 100, where F is the frequency of *Botryosphaeriaceae*; NBot is the number of fragments from which *Botryosphaeriaceae* were isolated; and NTot is the total number of woody fragments from which fungi were isolated [33].

2.4. DNA-Based Identification

All fungal morphotypes were transferred onto sterile cellophane in 9 cm Petri dishes containing PDA and maintained in the dark at 24 ± 1 °C for 1–2 weeks. Approximately 80 mg of mycelium was harvested from the cellophane surface for each fungal morphotype and placed in a sterile 2 mL Eppendorf tube. DNA was extracted using a GenElute plant Genomic DNA Miniprep kit using standard protocol (Sigma Aldrich, St. Louis, MI, USA) and, finally, stored at −20 °C. Amplification of the Internal Transcribed Spacer ITS region (including spacers ITS1 and ITS2 and the internal 5.8S rDNA gene) was conducted using the universal primers ITS1/ITS4 designed by White et al. [34] and applying PCR mixture and conditions as described in Moricca et al. [35]. Amplification products were visualized by putting 5 µL of each PCR amplicon in a gel electrophoresis containing 1% agarose gel (Sigma-Aldrich), 1× Tris-acetate-EDTA (TAE) buffer and ethidium bromide (0.5 µg mL^{-1}) as staining. The approximate size (bp length) of the amplification products was determined by adding to the gel the 100 bp DNA ladder Ready to Load (Genespin). PCR amplicons were purified by using the Kit NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. Purified products were sent for forward and reverse sequencing to StarSEQ® GmbH (Mainz, Germany). The nucleotide sequences were read and edited with FinchTV version 1.4.0 (Geospiza, Inc.; Seattle; WA, USA, http://www.geospiza.com/finchtv, accessed on 3 October 2023). The identity of analyzed fungi was determined by comparing the obtained consensus sequences with that deposited on NCBI by applying the nucleotide BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST accessed on 28 September 2023) searches [36]. Generated sequences were submitted and deposited in NCBI GenBank (Table 2). Sequences were aligned with ClustalX v. 1.83 [37] using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25%).

Table 2. Number of *Botryosphaeriaceae* species isolated from *F. ornus* at the investigated sites and GenBank accession numbers of one representative isolate per species.

Species	No. of Isolates	Sites	ITS GenBank Code
Botryosphaeria dothidea	53 [a] + 21 [b]	1–20; 22–24	OR119872
Diplodia fraxini	42 [a] + 15 [b]	1–5; 9–22; 25	OR177960
Diplodia subglobosa	3 [b]	19; 24	OR805517
Dothiorella iberica	2 [b]	18	OR805518
Dothiorella omnivora	2 [b]	21	OR805519
Neofusicoccum parvum	34 [a] + 3 [b]	2; 4; 7–9; 11–15; 19; 23	OR835588

[a] University of Florence collection. [b] University of Padua collection.

ITS sequences of representative isolates were edited and aligned in a dataset with 9 other sequences (ex-type sequences of 9 species belonging to 4 genera: *Botryosphaeria*, *Diplodia*, *Dothiorella* and *Neofusicoccum*) available in GenBank. Maximum likelihood (ML) analyses were performed with MEGA-X 10.1.8, including all gaps in the analyses. The best model of DNA sequence evolution was determined automatically by the software [38].

2.5. Pathogenicity Tests

Pathogenicity tests were conducted in an experimental field-plot at G.E.A. Green Economy and Agriculture, Centro per la Ricerca s.r.l., Pistoia, Tuscany (43.9192134° N, 10.9071857° E; http://www.cespevi.it, accessed on 1 June 2021) on 120 2-year-old seedlings of *F. ornus*. The experiment was performed from June to September 2021. Seedlings were potted using commercially produced loam and maintained in field conditions (outdoors) at 12 to 38 °C with drip irrigation in rows 30 cm apart. Inoculations were performed with three representative isolates of the most frequent fungal species (*B. dothidea*, *N. parvum* and *D. fraxini*) in the lower part of the stem, about 25 cm from the base of each seedling (about 1.2 cm in diameter). Thirty seedlings were used for each fungal species and 30 seedlings were mock-inoculated using a sterile PDA plug as control. The inoculation point was disinfected on each seedling with 90% ethanol, and a 6 mm diameter cork borer was used to remove the bark and expose the cambium. A 5 mm plug of mycelium of the tested fungal species, grown in Petri dishes in the dark on 2% PDA for 7 days at 25 °C, was inoculated in each seedling, with the mycelium side placed downwards into the wound. Mycelium was then covered with cotton soaked in sterile water and parafilm. To satisfy Koch's postulates, at the end of the trial, seedlings were taken to the laboratory, and re-isolations were performed from all the inoculated and control plants. Re-isolated fungal cultures were identified at species level by morphological and DNA-based analyses (ITS region amplification and sequencing). The pathogenicity of each tested fungal species was assessed by measuring the length of lesions (mm) developed on each seedling. Inspections, with lesion measurements, were conducted every two weeks.

2.6. Statistical Analyses

Pathogenicity test data were statistically analyzed by applying an analysis of variance (ANOVA). Significant differences between mean values were determined using Fisher's least significant differences (LSD) multiple range test ($p = 0.05$) after one-way ANOVA using the software SPSS V.28 (IBM Corporate, Endicott, NY, USA). Differences achieving a confidence level of 95% were considered significant.

3. Results

3.1. Field Surveys

Symptoms were found on a total of 91 *F. ornus* plants and were visible on shoots, branches and stems. *F. ornus* plants were affected both in the adult and the juvenile stages, but the severity of the disease was greatest for the young regeneration, with high sapling

mortality (Figure 1A–C). On adult trees, typical *Botryosphaeria* cankers were observed on the main stem (Figure 1D–F). Cankers on the stem appeared as swollen lesions with bark cracks (Figure 1D,F), or as sunken, cracked bark areas (Figure 1E) presumably depending on the individual tree's readiness/capacity to respond to the infection with callus. When infection girdled the stem, the upper part of the crown died back.

Figure 1. *Fraxinus ornus* individuals with symptoms of decline/dieback in the field (**A**–**F**); scale bar = 5 cm. Mortality of natural regeneration, with evident crown desiccation and cankers on the stem (**A**–**C**). A canker on the stem originating at a dead branch, with discolored bark and callus formation (**D**); sunken canker with cracked areas girdling the stem (**E**); a swollen canker with cracked areas on the bark (**F**).

Debarking of cankered parts of trees revealed the underlying necrotic lesions of the inner bark (secondary phloem) (Figure 2A–D).

Figure 2. Visual representation in sequence of a longitudinal canker on a *Fraxinus ornus* trunk (**A**), subjected to progressive removal of the outer bark (**B**–**D**) to show the extension of the necrotic tissues throughout the inner bark (secondary phloem); scale bar = 5 cm.

The initial stages of the cankers were more easily observable on saplings, and they appeared as small, sunken, pale brown-purplish necroses (Figure 3A). Lesions then extended longitudinally, giving rise to narrow, elongated cankers that killed the sapling as soon as they girdled the stem (Figure 3B–D). More sporadically, cankers were observed on lateral branches of adult trees; as with the young regeneration, the distal part of the branch, beyond the canker, was dead (Figure 3E). On some saplings, cankers were diffused along the axis, revealing the ability of the causal agent to spread rapidly along the stem (Figure 3F,G). The multiple cankers caused dieback and death in most of the saplings and young trees.

Leaf and shoot blight with crown dieback were also frequently observed (Figure 4A–E). *B. dothidea* was consistently isolated from cankers (more than 95%) on the stem of young and adult *F. ornus* individuals; *N. parvum* was isolated only sporadically (approximately 5% of cankers). *D. fraxini* was isolated at very high frequency (90%) from green, succulent shoot tissues and foliage, while approximately 10% of these tissues were infected with *N. parvum* (8%) or *B. dothidea* (2%).

3.2. Isolate Identification

One hundred and sixty-five fungal colonies were identified based on morphological and molecular characteristics. The morphological traits of the isolates, including the morphology and size of the conidia and shape of the colonies, were determined on MEA (Malt Extract Agar) and PDA, according to standard procedures. The colony of *B. dothidea* was grey or dark brown with a sparse aerial mycelium and a cottony surface texture. Conidia were fusiform or irregularly fusiform, base rounded, hyaline, and unicellular, rarely forming a septum. *D. fraxini* showed a white colony at first and dark grey later; conidia were hyaline, aseptate, smooth, and oblong to ovoid with a broadly rounded apex. The colony of *Neofusicoccum parvum* was initially white, becoming a greyish aerial mycelium typical of *Botryosphaeriaceae* species. Conidia were hyaline, unicellular, and ellipsoidal-shaped, with an obtuse apex and truncated base. *B. dothidea* was isolated from 23 out of the 25 sites, occurring in 74 of the 91 *F. ornus* plants investigated in total. *D. fraxini* was isolated from 57 of the analyzed *F. ornus* plants, while *N. parvum* was found in 37 of the total plants (Table 2). *B. dothidea* and *D. fraxini* pycnidia were also observed on branches with cankers by stereomicroscopic observations.

Figure 3. *Fraxinus ornus* individuals with symptoms of decline/dieback in the field (**A**–**G**). Incipient canker on a young sapling, with light brown to purplish-brown bark; individual lesions are coalescing to form a large necrotic patch (**A**), scale bar = 2 cm. More advanced, depressed canker, with beige-brown bark in advancing (outer) edge and dark-brown in its central portion (**B**), scale bar = 2 cm; Ellipsoidal canker with longitudinal cracks and occasional small black fruiting bodies (pycnidia) (circle) protruding through the bark (**C**), scale bar = 2 cm; Typical, sunken, light-brown canker with a marked edge is girdling the stem of an *F. ornus* sapling (**D**), scale bar = 2 cm; lateral branch of an adult *F. ornus*, desiccated in its distal portion due to a canker (arrow) (**E**), scale bar = 20 cm; young *F. ornus* tree with multiple cankers along the stem (**F**), scale bar = 4 cm; detail of cankers spread along the axis of a young *F. ornus* tree sapling seen at higher magnification (**G**), scale bar = 4 cm.

Figure 4. Symptoms on branches and shoots of young *F. ornus* in the surveyed areas (**A**–**E**). Shoot folding and initial stage of dieback of a young individual caused by *D. fraxini*; a longitudinal necrosis (red circle) is visible on the succulent, non-lignified stem (**A**), scale bar = 2 cm; higher magnification view (arrow) of the longitudinal, dark lesion on the stem of the individual in A (**B**), scale bar = 1 cm; dieback and necroses of shoots and tips on young *F. ornus* individuals (**C**–**E**), scale bar = 2 cm.

The other three species, *Diplodia subglobosa*, *Dothiorella iberica* and *Do. omnivora*, were isolated from a limited number of samples in only one or two sites (Table 2). DNA sequencing confirmed the identification of representative morphotypes as belonging to *B. dothidea* (OR119872), *D. fraxini* (OR177960), *D. subglobosa* (OR805517), *Do. iberica* (OR805518), *Do. omnivora* (OR805519) and *N. parvum* (OR835588) with BLAST searches that revealed complete (100%) homology of isolates with those of the above species deposited in the GenBank database. ITS-generated sequences were edited and aligned together with representative isolates of *B. dothidea*, *Neofusicoccum luteum*, *N. parvum*, *Diplodia corticola*, *D. mutila* and *D. fraxini*. The isolates clustered in well-supported clades (ML bootstrap > 95%) together with sequences of ex-type cultures (Figure 5).

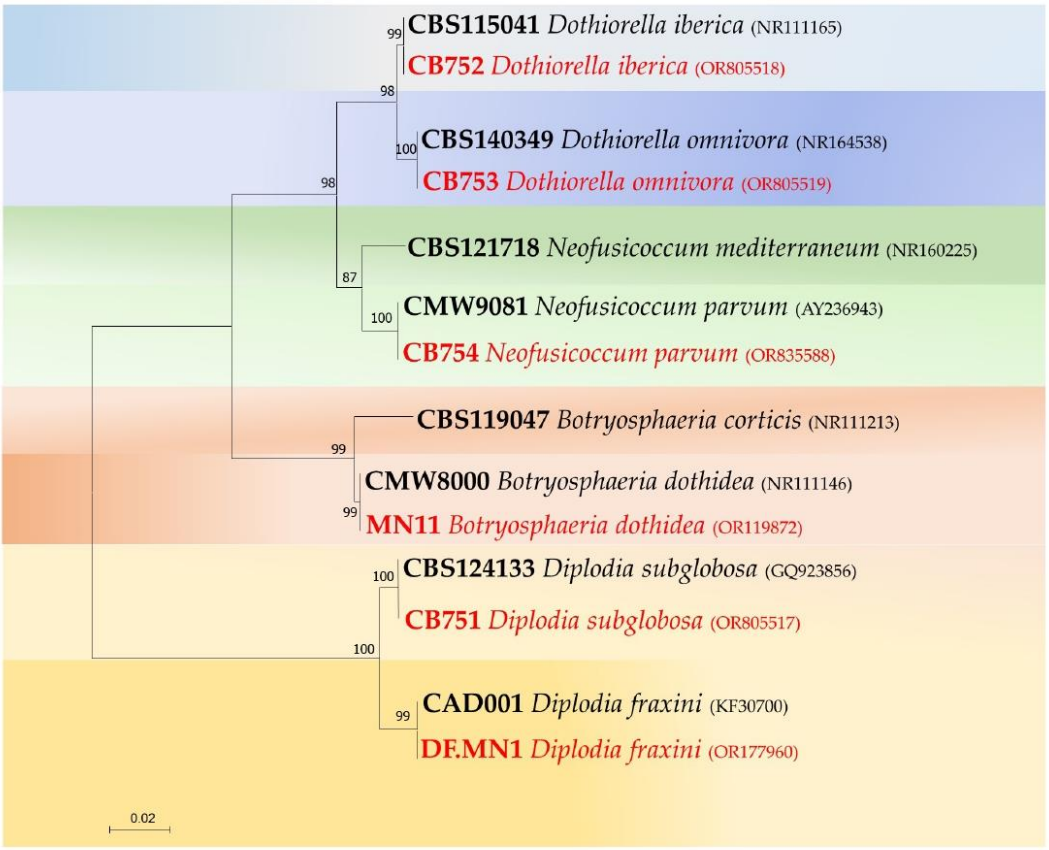

Figure 5. Maximum likelihood tree obtained from Internal Transcribed Spacer (ITS) sequences of *Botryosphaeriaceae* species. The tree is drawn to scale (0.01), with branch lengths measured in the number of substitutions per site. Bootstrap support values in percentage (1000 replicates) are given at the nodes. Ex-type cultures are in bold, and isolates obtained in this study in red.

3.3. Pathogenicity Tests

At the end of the pathogenicity trials, all *F. ornus* seedlings inoculated with *B. dothidea*, *D. fraxini* and *N. parvum* showed cankers and necrosis of different length around the infection court (Figure 6). By debarking cankered areas, dark brown inner lesions and internal discolorations were noticeable, corresponding precisely to the external lesions (Figure 6B). *B. dothidea* turned out to be the most aggressive pathogen, causing the larger cankers and under bark lesions (average length 58 ± 4.53 mm); *D. fraxini* caused lesions of 33 ± 1.72 mm; *Neofusicoccum parvum* caused less severe symptoms (mean lesions length = 22 ± 1.89) (Figure 7). With all the inoculated fungal species, lesion lengths were significantly greater than those recorded on control seedlings. Wounds appeared completely healed or with very small lesions at the mock-inoculation points on control seedlings (mean lesion length = 8 mm). The frequency of re-isolation of the three *Botryosphaeriaceae* species was 100%; no pathogenic fungal species were retrieved from *F. ornus* control seedlings. The identities of the reisolated species were confirmed by cultural, microscopic and molecular identification, thus fully fulfilling Koch's postulates. The pairwise comparison obtained from one-way ANOVA

showed significant differences ($p < 0.05$) between the three species and the control concerning lesion lengths and internal discolorations.

Figure 6. (**A**) Measurement of the extension of a canker artificially produced by inoculation with *B. dothidea* on an *F. ornus* seedling. A further canker, developed in the upper part of the image (arrow), proves the ability of the pathogen to spread rapidly along the stem, despite the seedling vigorously resisting infection, as is evident from the callus thickness. (**B**) Bark removal of cankered areas in the same seedling to observe the extent of the inner lesion highlighted the necrosis of the inner cambium, which was more pronounced in correspondence to the cankers (arrows), scale bar = 1 cm.

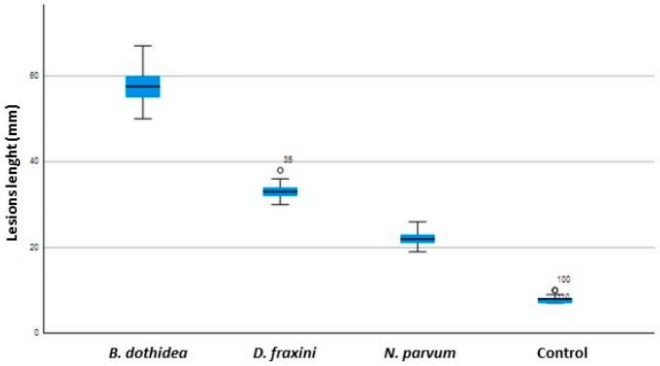

Figure 7. Graph depicting the extent of lesions produced on seedlings by artificial inoculation with *B. dothidea*, *D. fraxini* and *N. parvum*, compared to the control. Boxes represent the interquartile range, while the horizontal line within each box indicates the average value.

Some of the infected seedlings, even those inoculated with the virulent *B. dothidea*, reacted vigorously to infection by forming a thick wound periderm around the infection

court in order to promptly heal the wounds (Figure 6). Despite the vigorous reaction of the seedling, *B. dothidea* was able to spread rapidly upwards and downward along the axis—as already observed in the field—producing further cankers at a distance from the inoculation point (Figure 6A,B, arrows).

4. Discussion

The investigations conducted in this study provide compelling evidence that the dieback affecting *F. ornus* in forest formations of north-central Italy is to be ascribed to the action of prominent members of the *Botryosphaeriaceae* family. Symptoms similar to those caused by *Botryosphaeriaceae* on *F. ornus* have been reported on a plethora of woody hosts (fruit crops, tree plantations and natural forests) all over the world [22,23,29,39–41]. Although the *Botryosphaeriaceae* have been known as agents of diseases for some time, reports on their occurrence and deleterious impact on agricultural and forest systems have burgeoned in the last few decades [34,42,43]. This rise in *Botryosphaeriaceae* is probably due to multiple factors. First of all, there is the availability of more refined diagnostics tools enabling fungal taxa to be accurately identified, including cryptic and sibling species, also in those taxonomic groups, like the *Botryosphaeriaceae* family, with a taxonomic and nomenclatural history punctuated by confusion and controversy [22,23,44]. Global trade, especially the transnational movement of plant propagation material, has certainly played a key role in promoting the dispersal of some important members of this family into new, uncontaminated areas [45]. Anthropogenic disturbance, i.e., degradation fires, crop conversions, deforestation and inappropriate silvicultural interventions in general, by modifying agro-ecosystem processes and depleting host carbon reserves, make trees more vulnerable, thus more prone to infection and pervasive colonization by latent, opportunistic pathogens like the *Botryosphaeriaceae* [27]. However, most of the studies are today concordant in recognizing climate change as the major driver of phytopathological constraints induced by botryosphaeriaceous fungi [21]. Indeed, it is known that these fungi can live asymptomatically for an undefined period of time as endophytes inside plant tissues, switching to a pathogenic lifestyle when stressful environmental conditions, above all water stress, cause physiological impairment (e.g., reduced water transport across the apoplast) to their hosts [24–26,46]. Episodes of climate-related physiological damage to trees are becoming more and more frequent in the Mediterranean region (in which the area investigated in this study lies), which is considered today one of the most threatened by climate change. In fact, in the Mediterranean basin, the predicted global climate change scenario appears to be even more exacerbated, with warmer conditions, increased water deficits, heat waves and prolonged droughts being expected to increase over time [17–20]. The physiological stress caused to trees by climate vagaries and unusual weather events (included hail damage) is thus a precursor of massive tree infection by the *Botryosphaeriaceae* [47].

Studies dealing with *Fraxinus* dieback in Europe, including the Mediterranean areas, have mainly focused on assessing the pathogenic role of the ascomycete fungus *H. fraxineus* [4–7]. Only recently, this and a few other investigations have started taking into consideration the possible role of fungi other than *H. fraxineus*, among which the *Botryosphaeriaceae*, in the onset of ash decline/dieback [10,31,32]. Here, we provide evidence that six important fungi of the *Botryosphaeriaceae* family attack trees or parts of trees that are injured or in a weak or stressed condition. Three of these species in particular, namely *Botryosphaeria dothidea*, *Diplodia fraxini* and *Neofusicoccum parvum*, were consistently isolated from dead and dying *F. ornus* individuals. Among these, *B. dothidea* was isolated with the highest frequencies from stem wounds and was capable of producing the largest lesions in pathogenicity tests. *B. dothidea* grew in the living bark (phloem) and wood (xylem) and killed the branch or tree by girdling. The lower isolation frequency of the other two *Botryosphaeriaceae* species, taken together with the lower virulence they displayed in artificial inoculation tests, suggest that they are probably not primary agents of disease, although they are contributing to the overall decline of *F. ornus* trees. The high adaptation of *B. dothidea* is proven by its broad geographical distribution (it is a cosmopolitan fungus)

and its wide host range, continuously updated and expanding [24,26]. Hence, adverse environmental conditions like those caused by climate change can favor this fungus in attacking and infecting hosts previously uninfected, creating a greater impact and potential expansion in different parts of the world [15,48–58]. *D. fraxini* has already been reported in studies on the etiology of diseases affecting *Fraxinus* [32]. Data on the current distribution of this fungus are still lacking also due to the recent taxonomic re-classification that has reassigned the name *D. fraxini* to this fungus, previously included in the *D. mutila* complex [10,59]. The adaptability of this fungus has been demonstrated by its reports from sites with different climatic conditions (average annual temperature from 6.1 to 15.1 °C and average annual precipitation ranging from 650 to 2050 mm), a fact that confirms the plasticity of this fungus against environmental changes [31]. *N. parvum* is a generalist, latent pathogen distributed worldwide, infecting a number of hosts in both fruit crops and native vegetation [60–64]. The widespread occurrence of this fungus reflects either its migration through the man-mediated movement of infected plant germplasm or its own ability to spread into wild or managed (e.g., plantations) ecosystems [41,65]. Although appearing more sporadic, the other three species are well known as pathogens involved in ash dieback [31]. *D. subglobosa*, in particular, plays a key role in the decline of common ash in north-eastern Italy and Slovenia [66]. As with *Diplodia fraxini*, this species was considered for a long time as belonging to the *Diplodia mutila* complex [31].

5. Conclusions

The serious problem of ash dieback by *H. fraxineus* has been misleading and has led to limitations in diagnostic and epidemiological investigations in some parts of Europe. This study adds essential knowledge to the etiology of *Fraxinus* decline/dieback and provides basic information on pathogenic fungi affecting *F. ornus* in the Mediterranean area. Here, fungi from the *Botryosphaeriaceae* family play a prevalent role in the development of the phenomenon, with *B. dothidea* and *D. fraxini* proving to be prevalent. These findings can have implications for future research and practice, better directing diagnostic efforts and monitoring campaigns.

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Chapter III

Trunk injection delivery of biocontrol strains of *Trichoderma* spp. effectively suppresses nut rot by *Gnomoniopsis castaneae* in chestnut (*Castanea sativa* Mill.)

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Trunk Injection Delivery of Biocontrol Strains of *Trichoderma* spp. Effectively Suppresses Nut Rot by *Gnomoniopsis castaneae* in Chestnut (*Castanea sativa* Mill.)

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Simple Summary: The cultivation of chestnut trees for fruit production has historically played a fundamental role in the survival of the populations of the poorest and most disadvantaged mountain areas of Southern Europe. Starting from the 2000s, a new fruit parasite, the fungus *Gnomoniopsis castaneae*, agent of the brown or chalky nut rot, has put chestnut cultivation in crisis. The control of this pathogen in the forest is difficult due to its endophytic lifestyle, wide distribution and the inability to resort to chemical control, given the need for environmental protection. In this study, strains of three *Trichoderma* species, *T. viride*, *T. harzianum* and *T. atroviride*, were tested for their ability to inhibit *G. castaneae*, both in the forest and in vitro. The inoculation of the antagonists was in the stem of adult chestnut trees using endotherapy, in two consecutive years. Statistically significant results demonstrated that the three biocontrol agents effectively suppressed nut rot in both chestnut stands and in vitro tests. Endotherapic treatments have proven to be an innovative and effective solution for the biological control of this emerging disease.

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Abstract: *Gnomoniopsis castaneae* is responsible for brown or chalky nut rot in sweet chestnut (*Castanea sativa*), causing heavy reductions in nut production. Controlling it is challenging, due to its inconspicuous infections, erratic colonization of host tissues and endophytic lifestyle. Fungicides are not applicable because they are prohibited in chestnut forests and strongly discouraged in fruit chestnut groves. *Trichoderma* species are safe and wide-spectrum biocontrol agents (BCAs), with a variety of beneficial effects in plant protection. This study tested selected strains of *T. viride*, *T. harzianum* and *T. atroviride* for their ability to suppress *G. castaneae*. Field experiments were conducted in four chestnut groves (two test plots plus two controls) at two sites with a different microclimate. As the size of the trees were a major drawback for uniform and effective treatments, the *Trichoderma* strains were delivered directly by trunk injection, using the BITE® (Blade for Infusion in TrEes) endotherapic tool. The BCA application, repeated twice in two subsequent years, significantly reduced nut rot incidence, with a more marked, presumably cumulative, effect in the second year. Our data showed the tested *Trichoderma* strains retain great potential for the biological control of *G. castaneae* in chestnut groves. The exploitation of *Trichoderma* spp. as biopesticides is a novelty in the forestry sector and proves the benefits of these microbes in plant disease protection.

Keywords: *Castanea sativa*; nut rot; biocontrol; endotherapy; precision crop protection (PCP); sustainability

1. Introduction

Chestnuts are multi-purpose tree species, domesticated as crops since ancient times in various parts of the world, supplying nuts, timber, tannins and, indirectly, flour and

honey [1]. Among these species, the sweet chestnut (*Castanea sativa* Mill.) has, for centuries, played a key role in many European countries as a primary source of livelihood for people living in mountainous areas [2].

In Italy, the second biggest producer of chestnut fruits in Europe (approximately 300,000 t in 2022 according to an FAO report) [3], the cultivation of sweet chestnut began to decline in coincidence with rural depopulation, an effect of the process of industrialization, which came with an unmanageable trend towards urbanization [4]. This abandonment of chestnut cultivation, essentially due to socio-economic development, was further accentuated starting from the period around World War II, this time due to biological factors, i.e., the advent of new diseases and pests. Among these are the introduction of the harmful chestnut blight pathogen *Cryphonectria parasitica* (Murr.) Barr.; the recrudescence of old infections by *Phytophthora cambivora* [(Petri) Buisman], the oomycete agent of "ink disease"; the more recent arrival of the cosmopolitan, plurivorous root rot pathogen *Phytophthora cinnamomi* Rands; as well as the introduction, in the last 20 years, of the Asian chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu. To make matters worse, biotic stresses were compounded by abiotic disorders, the major driver of which is climate change; climate warming, with high temperatures, low humidity and unusual weather events (e.g., increased recurrence of heat waves and drought during the growing seasons) cause severe defoliation and fruits abortion or non-ripening on chestnuts, with harmful consequences to chestnut cultivation and local economies [5].

The long-term crisis of chestnut cultivation, due to the depopulation of mountainous territories, the succession of parasitic attacks and climate anomalies [6] has shown, however, a marked reversal of trend in the last few years [7]. In recent years, in fact, chestnut cultivation has acquired a new and significant role, brought about by a new appreciation of the health and nutritional qualities of the fruit, as well as of the nutraceutical values of chestnut flours [8]. Furthermore, chestnut formations are being revalued enormously, due to a rediscovery of the range of ecosystem services these stands are able to offer, including their high landscape and cultural value, with an awakening of the demand from citizens for mountain traditions and environmental awareness [9].

Fruit spoilage by diseases, pests and abiotic factors was always one of the main causes of product loss in the chestnut production chain, but normally a low, tolerable percentage of fruits was affected in storage [10]. This situation changed drastically with the advent of a new fungal disease of the fruit. The disease was reported for the first time almost contemporarily in Australia and Northern Italy around 2012, with two different fungal species, now considered synonyms, which were recognized at that time as the causative agents: *Gnomoniopsis castaneae* Tamietti and *Gnomoniopsis smithogilvyi* L. A. Shuttleworth, E. C. Y. Liew, and D. I. Guest [11]. The fungus, belonging to the *Gnomoniaceae*, induces nut rot, with alterations in the internal tissue and organoleptic features of the fruit, which become white-brown in color, chalky and sponge-like in texture and takes on an unpleasant taste, becoming inedible. The heavy product losses caused by this pathogen today represent the most serious threat to chestnuts production [12]. Reports of the presence of this fungus are referred mainly to post-harvest investigations, with disease incidences that can vary depending on climate trend and environmental conditions of the growing areas but do not rarely reach 90% [13]. Besides causing nut rot in *Castanea* spp., this ascomycete was also reported to cause cankers on seedlings and young chestnut trees. It is also found as an endophyte in asymptomatic tissues of various tree and shrub species, including *Fraxinus ornus*, *Quercus ilex*, *Quercus cerris*, *Corylus avellana*, *Pinus pinaster* and *Buxus sempervirens* [14].

Due to the inconspicuous infections and the endophytic colonization of internal tree and nut tissue by *G. castaneae*, control measures against this harmful pathogen are, to date, limited and often ineffective [15]. Currently, most of the control methods rely on water curing, sterilization and the application of chemicals post-harvest [10,16]. However, the efficacy of these methods is strictly linked to disease severity, often being ineffective with high infection levels. The application of fungicides in chestnut stands, on the other hand, raises concerns for environmental problems, linked to their deleterious impact on

non-target organisms and the emergence of pathogens' resistant strains [6]. These effects, coupled with the risk chemical applications pose to human health, have led authorities to ban the use of pesticides in forest environments. Hence, alternative solutions that are effective against *G. castaneae* are highly demanded.

The exploitation of the natural microbial enemies of plant pathogens as biocontrol agents (BCAs) is a new, promising strategy for sustainable crop protection, strongly promoted in Europe by national and EU agricultural and environmental policies [17]. Indeed, biocontrol enables the effective and environmentally friendly control of plant diseases by harnessing the natural ability of certain microorganisms to antagonize phytopathogens through various mechanisms. These mechanisms include competition for space and nutrients, direct parasitism, production of antimicrobial compounds and interaction with the plant system. This interaction enhances host growth and resistance by activating both ISR (induced systemic resistance) and SAR (systemic acquired resistance) pathways [18]. BCAs can be selected based on the targeted host–pathogen system among different organisms including fungi, bacteria and viruses that can be found in the ecosystem but among these, some genera (e.g., *Agrobacterium*, *Ampelomyces candida*, *Bacillus*, *Coniothyrium*, *Pseudomonas*, *Streptomyces* and *Trichoderma*) have acquired great importance, being marketed and promoted worldwide due to their BCA effectiveness [19]. Fungi of the genus *Trichoderma* have received much interest from both science and the commercial market, having been recognized as potential BCAs since the 1930s [20]. *Trichoderma* species are soilborne, free-living fungi that, depending on the strain and species, can effectively compete with naturally occurring microorganisms, restricting their growth for weeks or months. In many plant/pathogen/microbial antagonist interactions, these beneficial fungi revealed the ability to suppress pathogen growth in the rootsphere, endosphere and phyllosphere, either by direct mycoparasitism or indirectly, by releasing antifungal compounds (e.g., antibiotics) [21]. The use of *Trichoderma* species as BCAs, biostimulants and biofertilizers has become widespread in agricultural and horticultural systems, relying on different methods of application that range from seed coating to post-harvest and from soil to foliar treatments [22].

Contrary to a now widespread application in agriculture, biocontrol is still rarely applied in forestry, with the few attempts to apply BCA-based control referring mostly to lab, glasshouse or nursery investigations [23,24]. Schubert et al. [25] tested the use of *Trichoderma* spp. for controlling wood decay fungi in adult trees, obtaining effective results. However, the application of biocontrol in forests is particularly challenging due to the following several drawbacks: the steepness of the territory (for the effective execution of treatments with machinery), the absence of roads, and the large size of trees. All these difficulties can make a biopesticide treatment inconspicuous, erratic or incomplete, not to mention that some treatment types (e.g., foliar applications) would cause a large waste of the BCA due to drift and its dispersal into the environment, and this could negatively affect the ecological resilience of forest ecosystems, creating further damage [26]. The application of biocontrol agents by endotherapy could overcome some of these issues, by not provoking dispersal of the injected BCA into the environment and strongly reducing (practically zeroing out) the doses needed for treatment. The efficacy of endotherapic treatments coupled with the use of *Trichoderma* spp. has already been demonstrated by Berger et al. [27], who tested the method against *Phytophthora* species on *Quercus robur* and *Fagus sylvatica*. The aim of this study was to assess the effectiveness of some *Trichoderma* species for controlling *G. castaneae* rot in chestnut fruits, by delivering selected strains of these BCAs in cultivated *C. sativa* trees by trunk injections, using a minimally invasive technique. The test was repeated twice, in two consecutive growing seasons (2021 and 2022).

2. Materials and Methods

2.1. Study Sites

The study was conducted in chestnut groves at localities "Ribuio" and "Bandina", in the Municipality of Ortignano-Raggiolo (AR), in the Casentino Valley (Arezzo, Tuscany),

in central Italy. These localities were about 1300 m apart from each other, in two separate valleys with different microclimatic conditions (Ribuio test plot 43.683688 11.704197 690 S; Ribuio control plot 43.682456 11.705163 620 S; Bandina test plot 43.671514 11.700513 820 E; Bandina control plot 43.675462 11.705967 700 E (Figure 1). Two plots (ca. 0.4 Ha each), one per each locality, were established for the experimental trials. Two additional same-surface plots, homogeneous for tree cultivar and size, soil type and microclimate, were set up as a control in the immediate vicinity of each of the plots selected for treatments.

Figure 1. Aerial view of the four plots (Ribuio test, Ribuio control, Bandina test and Bandina control); the plots under investigation are delineated in green.

The "Ribuio" plot was on a skeleton in the form of outcropping rocks on a steep slope, in a small gorge, near a stand of *Pinus* sp. and was characterized by high humidity, due to the proximity of a river. It had a south-east exposure and an average altitude of 650 m a.s.l. (above sea level). The "Bandina" plot had no water course nearby, and the land was slightly sloping and was exposed to the east, with less outcropping rocks and fairly deep soil. The thermo-hygrometric conditions were therefore markedly different between the two plots (and in the adjacent control plots): "Ribuio" was more humid and colder; "Bandina" was hotter (because it was sunnier) and drier. The following parameters were recorded at each plot selected for treatments: number of trees, DBH (diameter at breast height: 1.30 m), height of each tree and age class (young < 30 years; adult 30–80 years; mature 80–150 years; overripe > 150 years height) (Table 1).

Table 1. Age class, DBH (cm) and height (m) of treated trees in each chestnut stand.

Ribuio Chestnut Stand			
Number	**Age (Y = Young, <30 years; M = Mature, 80–150 years; O = Overripe, >150 years)**	**Diameter at Breast Height DBH (cm)**	**Height (m)**
1	O	79.58	11.8
2	O	42.97	15.9
3	O	86.26	14
4	O	95.49	12.3
5	O	44.56	10
6	M	38.2	14.7
7	M	34.06	13.8
8	O	113.32	12.8
9	M	53.16	16.3
10	M	33.42	14.1
11	O	65.57	10.9
12	O	108.23	10.9
13	O	113	11.1
14	O	66.85	9.4
15	O	82.76	7.6
16	O	64.3	7.8
17	O	43.93	12.8
18	O	69.39	7.7
19	M	36.61	6.1
20	NA	28.87	NA
21	O	62.71	6.9
22	O	70.66	6.8
23	O	60.48	NA
24	NA	60.48	NA
25	O	63.66	NA
26	NA	49.34	NA
27	O	92.31	NA
28	O	98.04	NA
29	O	127.32	NA
30	M	49.34	NA
31	O	119.37	NA
32	O	93.9	NA
Bandina chestnut stand			
1	M	70.03	10
2	M	38.83	11.9
3	M	55.7	10.02
4	M	53.16	11.06
5	M	59.21	13.6
6	M	41.38	13.4
7	M	61.75	15.1
8	M	51.57	14.1
9	M	45.2	14
10	M	48.38	16.1
11	M	74.8	9.5
12	M	44.56	12.9
13	M	67.16	10.5
14	M	73.21	11.1
15	M	49.34	16.8
16	M	53.48	16
17	M	46.47	11.4
18	M	48.38	12.2
19	M	59.84	14
20	M	60.8	11.9
21	M	50.93	11.5

Table 1. *Cont.*

Ribuio Chestnut Stand			
Number	**Age (Y = Young, <30 years; M = Mature, 80–150 years; O = Overripe, >150 years)**	**Diameter at Breast Height DBH (cm)**	**Height (m)**
22	M	66.85	11.5
23	M	58.89	10.3
24	O	194.17	10.9
25	Y	30.24	13.6
26	Y	35.01	13.4
27	M	48.7	13.5
28	M	49.02	12.4
29	Y	29.92	16.3
30	M	59.21	14.2
31	M	37.24	14.5
32	M	42.34	14.7
33	M	40.11	14.3
34	M	61.75	15.6

NA = Not Available.

2.2. Soil Sampling and Trichoderma Isolation

A total of 20 soil samples were collected from the four study plots. Each area was divided into five homogeneous subplots, with one soil sample being collected from each subplot. Each sample was placed in a nylon bag, labeled and stored at 4 °C until use. For the isolation of *Trichoderma* from soil, modified malt extract agar (MEA, DIFCO, Detroit, MI, USA) was used. Six-fold serial dilutions of each soil sample were then prepared in sterilized, distilled water and 1 mL of diluted sample was poured onto the surface of the MEA, amended with 25 mg/L of streptomycin sulfate (Sigma Aldrich, Steinheim, Germany). The plates were incubated in the dark at 24 ± 1 °C for 7 days, according to the species requirements. Morphologically different colonies appearing on the plates were sub-cultured onto MEA and stored at 4 °C.

2.3. Identification of G. castaneae and Trichoderma spp. Strains

G. castaneae strains were isolated from infected nuts, whereas *T. viride* and *T. harzianum* strains were retrieved from soil samples. The appearance and morphology of the colonies (surface topography, texture, compactness, mycelium pigmentation and margin type) were determined on malt extract agar (MEA). For each morphotype, tufts of mycelium were picked off by scraping colony surfaces with a sterile dissecting needle, then mounted on glass slides in a drop of 0.5% KOH or Lactophenol for direct examination under the light microscope. Conidial micromorphology was determined under a Zeiss light microscope (ZEISS, Jena, Germany) at ×40 magnifications, by averaging 200 measurements per fungal taxon. Images were captured with an Optikam 4083.B5 microscopic Digital USBCamera operated with OptikaView version 7 acquisition software (OptikaSrl, Ponteranica, Italy). The identity of representative strains of each species was confirmed through sequence analysis of the region spanning the 5.8S rRNA gene and flanking Internal Transcribed Spacers 1 and 2 (ITS1-5.8 S-ITS2). For DNA extraction, fungal strains were grown on sterile cellophane in 90 mm Petri dishes containing 1% potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) and maintained in the dark at 20 °C. After 7 days of incubation, ca. 70 mg (fresh weight) of mycelium was scraped off the cellophane surface and stored at −20 °C in a 2 mL Eppendorf tube until use. Genomic DNA was extracted using the GenElute plant Genomic DNA Miniprep extraction kit (Sigma Aldrich, St. Louis, MO, USA), following the manufacturer's instructions, and stored at −20 °C. Internal transcribed spacer (ITS) region PCR amplification was performed on extracted samples by using ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) universal primers [28]. Cycling and sequencing conditions were as described in

Moricca et al. [29]. The identification of retrieved fungal taxa was undertaken by processing the relative sequences with the nucleotide–nucleotide BLAST® search tool (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST accessed on 11 October 2023). Generated sequences were submitted and deposited in NCBI GenBank database.

2.4. In Vitro Antagonism Tests

The *T. harzianum* and *T. viride* isolates retrieved from the soil were confronted with *G. castaneae* for their ability to inhibit the pathogen's mycelial growth (%) in in vitro dual culture assays. For comparison, the test also included a commercial strain (SC1) of *T. atroviride* obtained from a Vintec® biological commercial formula (Certis Belchim B.V., Utrecht, The Netherlands). Each test was performed in a 9 cm diameter Petri dish containing 12 mL of MEA. Two plugs (diameter, 4 mm) retrieved from 7-day-old colonies of *G. castaneae* and of each one of the selected antagonists, were inserted in each dish, 6 cm apart from each other. Mycelial interactions were determined according to Badalayan et al. [30]. The experiment was conducted with 10 replicates for each antagonist and for the control, represented by two isolates of *G. castaneae*. Dishes were incubated at 25 °C in the dark for 8 days. Mycelial radial growth was measured after 8 days in each dish, calculating the growth area of each fungal species using the ImageJ software (version 1.8.0). Results were then compared to those obtained for the controls. The inhibition index percentage (I%) was calculated as follows [31]:

$$I\% = \left[\frac{(RM - rm)}{RM}\right] \times 100$$

where *RM* is the radius of the *G. castaneae* colony in the control plate, and *rm* is the radius of the colonies in the direction of the antagonist.

Hyphal interactions between colonies were assessed at 7 and 14 days under an optical microscope (ZEISS, Jena, Germany), with the antagonistic ability of *T. viride*, *T. harzianum* and *T. atroviride* specifically determined following Badalayan et al. [30], using the rating scale described in Table 2.

Table 2. Rating scale of the interaction types among the antagonistic fungus and investigated pathogen, following Badalayan et al. [30].

Type of Interaction	Interaction	Value
A	Stop of colony growth by contact with mutual inhibition	1
B	Remote stop without mycelial contact	2
C	Growth of one colony over another without initial stop	3
CA1	Partial growth of one colony over another after contact arrest	3.5
CA2	Complete growth of one colony upon another after contact arrest	4.5
CB1	Partial growth of one colony upon another after remote arrest	4
CB2	Complete growth of one colony upon another after remote arrest	5

2.5. Biocontrol Agent Mixture Preparation

The solution used for endotherapic treatments was prepared by collecting *T. harzianum* and *T. viride* conidia from pure 7–10-day-old MEA cultures. For *T. atroviride*, the preparation was made according to the manufacturer's instructions for the Vintec® (Belchim Crop Protection, Londerzeel, Belgium) biological formulation. The collected fungal mixture was filtered through filter paper (Whatman® Grade 1, Buckinghamshire, UK, diameter: 9 cm, pore size: 11 µm) to remove mycelium fragments. The concentration of conidial suspensions was then adjusted to 10^8 conidia/mL of water by using a counting chamber. The final dose of the fungal mixture utilized in trunk injections was 0.8 mL/10 cm of trunk circumference, following Berger et al. [27].

2.6. In Vivo Tests

In order to investigate the ability of *Trichoderma* species to antagonize the nut rot agent *G. castaneae* in planta, selected strains of the BCAs were delivered by means of trunk

injections into the 66 adult chestnut trees growing in the two chestnut groves Bandina (34 trees) and Ribuio (32 trees) (see Table 1 above). Endotherapic treatments were performed at breast height (about 1.5/1.7 m from the ground) by using the BITE® (Blade for Infusion in TrEes) injection tool from De Rebus Plantarum (Vicenza, Italy). This instrument enables a targeted delivery of the biopesticide, without producing holes in the tree, but rather by causing a minimal (a few mm) vertical lesion, which the plant normally heals in a couple of weeks [32]. The *Trichoderma* solution was applied in June, when the trees were actively growing, with the crown fully expanded, and the full hydraulic tension of the transpiration flow guaranteed the maximum absorption of the solution (Figure 2). Treatments were performed during two subsequent growing seasons, 2020 and 2021.

Figure 2. Application of conidial suspensions of the BCAs on *C. sativa* stems with the BITE® injection tool.

2.7. Assessments of G. castaneae Incidence

Plants were monitored for their health conditions throughout the duration of the field experiment, with plant material (foliage, shoots, green curls with unripe nuts) being collected and analyzed periodically. Up to 400 fully ripened nuts were finally randomly collected from chestnut trees in each treated and untreated stand in October (Figure 3a,b). Fresh chestnuts were transported to the laboratory and stored at 4 °C before isolations. Each chestnut was surface washed with 75% ethanol (1 min) and 3% sodium hypochlorite (NaOCl) (3 min), then rinsed three times in sterile water. A sterile scalpel was used to remove the outer lignified shell and open the nut by cutting it in half. Five fragments (approximately 1 × 1 × 2 mm in size) were randomly excised from the tissues of each fruit and plated in 90 mm diameter Petri dishes filled with 2% MEA. All dishes were incubated in the dark at 25 °C for 3 days, according to species requirements. The presence of *G. castaneae* was analyzed for each dish by comparing macro- and micro-morphological features of each obtained mycelium with that of a *G. castaneae* isolate, whose identity was previously assessed by ITS sequencing (Figure 3c).

Figure 3. Symptoms caused by *G. castaneae* in nuts: (**a**) a fruit with a completely dehydrated endosperm; (**b**) a freshly collected nut and cut in the field displaying a spongy or chalky appearance with distinctive dark-brown lesions. This discoloration further underscores the altered composition, indicating potential physiological changes within the fruit; (**c**) a *G. castaneae* culture grown on malt extract agar (MEA) after two weeks.

2.8. Statistical Analyses

For all the traits considered, the means and standard deviations were calculated for all the combinations considered. A one-way ANOVA was performed to test the significance of the in vitro antagonistic activity of three *Trichoderma* spp. versus *G. castaneae*. All data refer to the four chestnut stands (two treated and two untreated). Homogeneity of variance and normality tests were performed using the Levene and Shapiro–Wilk tests. SPSS V.28 (IBM Corporate, Endicott, NY, USA) was used for the statistical analysis, with χ^2 square test, to identify significant differences among treatments. The square test was applied to analyze the significance of the differences between trees treated with *Trichoderma* spp. and the controls (with no BCA application). The incidence of *G. castaneae* was calculated for each treated and untreated stand as the ratio (%) between the number of affected nuts and the total number of nuts analyzed.

3. Results

3.1. Gnomoniopsis castaneae and Trichoderma spp. *Identification*

The colonies of *G. castaneae* were initially white, turning to light gray after 7 days of incubation. The mycelium appeared either flat or thick and densely woolly. Margins were diffuse and developed in concentric circles. In the innermost portion of the colony, acervuli, ranging in color from orange to red, developed after 10 days. They were either superficial or erupting on the upper surface, circular, solitary or gregarious. Conidia were hyaline, one-celled, ovoid–oblong, straight or curved. Colony phenotypes, as well the size and shape of conidia, matched exactly the original descriptions of Visentin et al. [33] and Shuttleworth et al. [34]. DNA sequencing confirmed the representative morphotype as belonging to *G. castaneae* (PP326312), with BLAST searches that revealed complete (100%) sequence homology with those of the pathogen already deposited in the GenBank database. *Trichoderma harzianum* exhibited a cottony white, slightly yellowish mycelium, with a flat growth profile. Colonies developed at 8 days showed several concentric rings with dark green conidial production. *Trichoderma viride* colonies showed a uniform appearance with light green-yellowish conidia evenly distributed across their surfaces (without differentiating concentric rings). Growth rates and cultural characteristics of the two *Trichoderma* species differed on the same medium at a constant temperature of 24 °C. *T. viride*, from all soil types, demonstrated the highest growth rate compared to *T. harzianum*. *Trichoderma harzianum* conidia were from globose to sub-globose, with a dark green color. *T. viride* presented globose conidia, with a color ranging from light yellow to green.

Sequence analysis confirmed the identities of *T. harzianum* and *T. viride* (GenBank acc. nos. PP326311 and PP326313, respectively), with BLAST searches that revealed complete (100%) homology with sequences of the two species retrieved from the GenBank database.

3.2. In Vitro Tests

A priori in vitro assays were useful for assessing *Trichoderma* species for their ability to inhibit *G. castaneae*. *T. viride* and *T. harzianum* strains were highly effective against *G. castaneae*. The parasitization of the pathogen was already visible at 96 h. After 8 days, *G. castaneae* mycelium turned out completely overgrown and replaced by strains of these two *Trichoderma* species (Figure 4a,b). The growth of *T. atroviride* at 25 °C was lower than that of *T. viride* and *T. harzianum*, with only a partial interaction with *G. castaneae* that was observed at the margin of the two colonies (Figure 4c). Hyphal interactions for *T. viride* and *T. harzianum* resulted in the CA2 subtype of Badalyan's scale; *T. atroviride* ranked as CA1 of the same scale. Statistical analyses confirmed the different behaviors/inhibitory effects of the three *Trichoderma* species. The one-way ANOVA statistical criterion showed that *T. atroviride*'s area value differed significantly after 6 days from that of the other two antagonistic isolates ($p > 0.05$). The highest inhibition capability was found for *T. harzianum* (99.14%), followed by *T. viride* (78.12%) and *T. atroviride* (51.82%) (Table 3).

Table 3. Antagonism of *T. viride*, *T. harzianum* and *T. atroviride* against *G. castaneae* assessed after 6 days by (A) average *Trichoderma* spp. area; (I) inhibition index percentage of *G. castaneae* mycelial growth; and (CI) competitive interactions.

Isolates	A (cm^2) *	I (%) *	CI **
Trichoderma viride	63.58	78.12 b	CA2
Trichoderma harzianum	63.58	99.14 c	CA2
Trichoderma atroviride	41.03	51.82 a	CA1

* Mean values (10 repetitions) followed by different letters indicate significant differences at $p < 0.05$. ** Badalyan rating scale [30]: CA2 = complete growth of one colony upon another after remote arrest; CA1 = partial growth of one colony over another after contact arrest.

Figure 4. In vitro dual cultures of *Trichoderma* species against *G. castaneae* after two weeks of incubation at 25 °C: (**a**) *T. viride*/*G. castaneae* interaction; (**b**) *T. harzianum*/*G. castaneae* interaction; and (**c**) *T. atroviride*/*G. castaneae* interaction.

3.3. Endotherapic Treatments in the Field

The field trials proved the effectiveness of treatments with *T. viride*, *T. harzianum* and *T. atroviride* in curtailing *G. castaneae*, with the pathogen's incidence being significantly reduced in the investigated chestnut groves. Data from the first and second year of treatments showed a reduction in *G. castaneae* incidence, with this trend increasing in the second year. In fact, in 2020, different percentages of *G. castaneae* incidence were obtained in the two treated chestnut groves, which were 24% and 22%, respectively, compared to the two control areas (46% and 48%). In the second year of treatment (2021), the incidence of the target pathogen was 23% and 11% in the treated plots and 63% and 40% in the in the control plots. The decrease in *G. castaneae* incidence between treated and untreated plots resulted in percentages of 26% and 22% in 2020 and of 40% and 29% in 2021 (Figure 5). The chi-squared statistical analysis confirmed the significant differences ($p \leq 0.01$) between treated and untreated chestnut groves during the two years of analysis. No other nut rot pathogens were isolated from the analyzed nut samples.

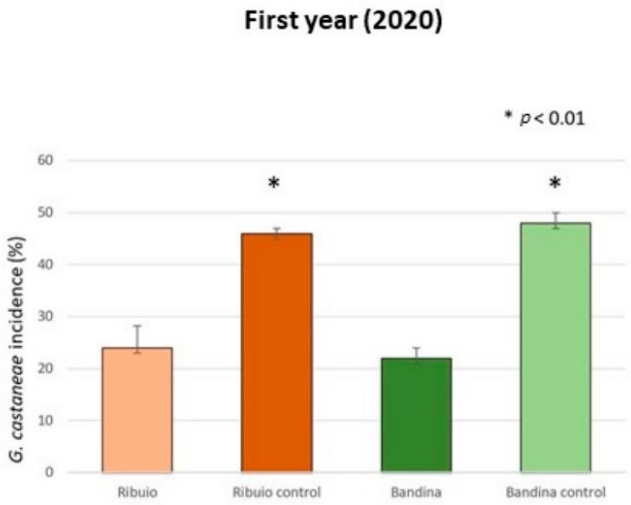

Figure 5. *Cont.*

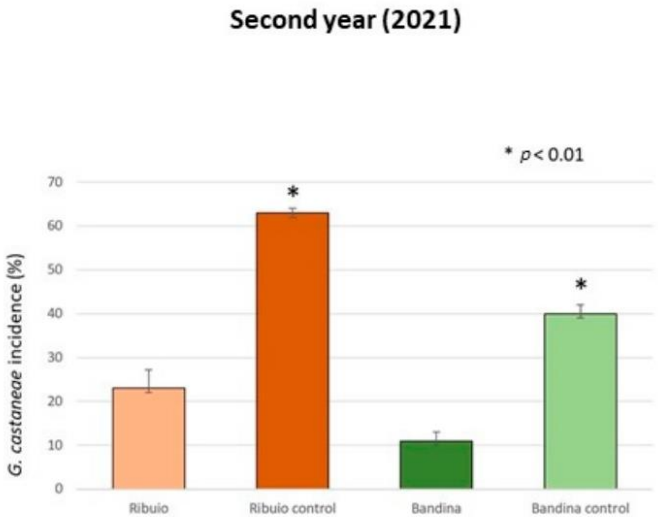

Figure 5. Average percentage of nut fruits found infected by *G. castaneae* in test plots at the "Ribuio" and "Bandina" chestnut groves and in their respective control plots. Black bars represent standard errors. * Significant differences between numbers of infected nuts found between localities (χ^2, $p < 0.01$).

4. Discussion

Nut rot by *G. castaneae* today represents the main cause of chestnut fruit deterioration. The disease strongly curtails nut production in the areas where it is present, the pathogen irremediably compromising nut quality and organoleptic features, making the fruit tissue chalky and inedible [11,13,35–39]. As the fungus can live as an endophyte in latency in chestnut organs and tissues, the application of effective control measures is troublesome and relies only on post-harvest treatments: water curing, sterilization and chemicals application [10,16]. Since a chestnut grove is considered in all respects a forest, chemical treatments are prohibited for environmental reasons. But even if they were not prohibited, chemical treatments would still be difficult to implement due to the morphology and slope of the terrain on which chestnut groves are often located, the environmental and spatial heterogeneity within the plantation and the large size of the trees. For all these reasons, in this work, biological control based on the application of *Trichoderma* species was evaluated as an alternative strategy for the control of *G. castaneae* in chestnut groves. The implementation of biological control against plant pathogens in agro-ecosystems by releasing competent *Trichoderma* strains is a safe technology that could solve issues related to environmental and health risks inherent with conventional control strategies [40,41]. Fungi of the genus *Trichoderma* are innate with the environment, being common inhabitants of soils, both agricultural and forest, and are often also found as endophytes in many plant species [42]. The effectiveness of *Trichoderma* species as BCAs against plant pathogens has been known for a long time and has been successfully applied in a variety of agricultural crops, especially in horticulture [43–49].

The application of biological control methods against forest pathogens, on the contrary, has been scarcely investigated and few attempts were made on adult trees [25,26]. In this work, endotherapy was exploited to deliver *Trichoderma* BCAs against *G. castaneae* directly in the stem of cultivated chestnut trees. Results were promising and consistent with those obtained by Berger et al. [27], who injected *T. atroviride* in *Quercus robur* and *Fagus sylvatica* using endotherapy as a preventive strategy against *Phytophthora* spp. infections. Indeed, our results showed that, in 2020, the incidences of *G. castaneae* were 24% and 22% in the

treated groves (Ribuio and Bandina), in comparison to their respective adjacent control plots (46% and 48%). A similar behavior was observed in the second year of treatment (2021), registering pathogen incidences of 23% and 11% in the treated plots (Ribuio and Bandina), and 63% and 40% in the control plots (Ribuio and Bandina). The decrease in *G. castaneae* incidence between treated and untreated stands resulted in percentages of 22% and 26% for 2020, and of 29% and 40% for 2021. The difference between the two locations, in the incidence of *G. castaneae* both in treated and in control plots, is ascribed to the different thermo-hygrometric conditions of the two sites. In fact, Ribuio is characterized by a particular microclimate: it is in a gorge, at the base of which a stream flows, which generates constant humidity and poor sunlight on the ground. Bandina, on the other hand, has a different exposure, is not located near streams and experiences high levels of sunshine. The particular temperature and humidity conditions of Ribuio may have favored *G. castaneae* infections.

The results of in vitro tests performed in this study have further confirmed the effectiveness of *Trichoderma* application as a biocontrol agent, as it inhibited *G. castaneae* in culture. Indeed, the inhibition of *G. castaneae* was observed by each tested *Trichoderma* species, with inhibition index percentages of 99.14% for *T. harzianum*, 78.12% for *T. viride* and 51.82% for *T. atroviride*. To the best of our knowledge, no other authors have reported a detailed assessment of the effects and interactions among *T. viride*, *T. harzianum* and *T. atroviride* on *G. castaneae* using dual culturing. Our in vitro results are in line with those of other authors who tested these *Trichoderma* species against different plant pathogens [24,50,51].

The treatment with three *Trichoderma* spp. was able to significantly reduce the necrotic surfaces of chestnuts caused by *G. castaneae*, and no systematic studies of resistance have been undertaken to date [11]. The results obtained in this study can also be compared with those reported by Pasche et al. [23], who applied *T. atroviride* against *C. parasitica* and *G. castaneae* by soaking chestnut scions in fungal propagule suspensions. These authors observed that the endophytic behavior of *Trichoderma* in chestnut tissues and the presence of *T. atroviride* were able to influence the infection of *G. castaneae*. Indeed, in agreement with our study, only 15% of treated scions analyzed by Pasche et al. [23] showed *G. castaneae* symptoms, while the percentage of *G. castaneae* symptoms in control scions was 75%. These authors ascribed the effectiveness of their biological control treatment to the possibility that inoculated *Trichoderma* had spread and colonized the totality of woody tissues.

The endotherapic method applied in this work in chestnut trees could improve the efficacy of biological applications, facilitating the entrance of *Trichoderma* in the plant vascular system by trunk injection. Endotherapy offers important advantages over other treatment methods: it lowers (reducing practically to zero) the dispersion of the BCA into the environment, and the doses needed for treatments are enormously reduced, making them more sustainable than traditional methods of application (e.g., spraying). The results obtained in this study suggest that the biological control method employed could represent a key advantage for chestnut growers, since it reduces the decay of fresh and processed nuts and the resulting economic losses.

Little is known about the duration of protection conferred by treatment with *Trichoderma* BCAs in forestry. However, the fact that in the second year a greater reduction in the incidence of rot was achieved means that there was an additive effect between the first and second year treatments. The mechanisms of action of *Trichoderma* strains (e.g., direct parasitism, lytic enzyme production, antibiosis, competition for nutrients and space) have been widely studied [18,19,21,22,50,52–54]. It is possible that the injected *Trichoderma* species utilized one or more of the above mechanisms, as well as also synthesizing bioactive compounds that elicited plant defense molecular responses, through the activation of either the ISR (induced systemic resistance) or the SAR (systemic acquired resistance) pathways. All these protection mechanisms may have restricted *G. castaneae*, conferring long-lasting defense to chestnut trees [18,41].

5. Conclusions

This is the first work in which BCAs of the genus *Trichoderma* have been administered on chestnut trees by endotherapy. The choice of endotherapic applications, dictated by technical–operational needs (we needed to administer the biopesticide in uncomfortable conditions, on large-sized plants and, last but not least, to reach the target pathogen in the most distal portions of the foliage), turned out to be a valid option. Further research is required to confirm the promising results obtained here and to better elucidate unknown aspects of *Trichoderma* mycoparasitism when conferring these BCAs to restrict *G. castaneae* in chestnut groves.

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General discussion

The decline of forests is a complex and multifactorial problem which is occurring with varying intensity in various areas of the planet. The phenomenon is often associated with Global Change, the effects of which are even more exacerbated in some areas in particular, like the so-called “Hotspots for Climate Change”. Given the extent of the phenomenon, and the economic and environmental losses associated with forest decline, the problem requires a timely and resolute response. Forests are in fact a fundamental tool for mitigating climate change, e.g. through carbon capture and oxygen production, but forest are also heavily affected by climate warming (Seddon et al., 2020). High temperatures, heatwaves, water deficits, extended droughts, and other extreme weather events are causing unprecedented stresses and mortality on forest trees, increasing the risk of fires, pathogen and pest attacks and land degradation (Lindner et al., 2014; Hartmann et al., 2022).

The stability and biodiversity of forests must also face threats from anthropogenic influences (Senf and Seidl, 2021). A pivotal factor contributing to imbalances in the natural environment is the unintentional introduction of exotic pathogens and pests (Liebhold et al., 2017; Panzavolta et al., 2021). Specifically, global trade is facilitating the movement of plant material across countries and continents, heightening the exposure of native plant communities to new pathogens and pests (Panzavolta et al., 2021). Human-mediated movement of plant germplasm and vegetable products enables these harmful agents to overcome the geographical barriers that historically played a role in shaping the distribution of living organisms within continents (Richardson et al., 2000).

Forest disturbances, like to the introduction and emergence of new pathogens, appears evident in various geographical areas worldwide. Notable, historical case histories are the pandemic spread of *Ophiostoma novo-ulmi*, the agent responsible for Dutch elm disease; the worldwide diffusion of *Seiridium cardinale*, the cause of a lethal canker disease on cypress and related *Cupressaceae* (Sache et al., 2011); the outbreaks induced by several *Phytophthora* species in oak forests; the epidemic spread of chestnut blight chestnut stands, ascribed to *P. cambivora*, *P. cinnamomi*, and congeneric species [(*P. cinnamomi* is currently causing severe oak mortality in Sardinia and Lazio regions (Scanu et al., 2014)]. But examples of devastating pathogens are also some recent diseases like the root and collar rots induced by *P. cryptogea* and *P. humicola* in plantations of *Pinus radiata* and *Pinus pinea* in central Italy; the root rot,

bleeding cankers and dieback of *Acer pseudoplatanus* caused by *P. acerina* (Ginetti et al., 2014); strawberry tree dieback by *P. parvispora* (Scanu et al., 2014); dieback and mortality of *Juglans* spp., in North America and, more recently, in Europe (in Italy, in particular) by the invasive ascomycete pathogen *Geosmithia morbida*, the agent of Thousand Cankers Disease (TCD) of Walnut and its vector the scolytid *Pityophthorus juglandis* (Moricca et al., 2019; 2020; Bracalini et al., 2023); the devastation of riparian alder formations in the Italian Alps provoked by the exotic rust *Melampsoridium hiratsukanum* (Moricca et al., 2021).

A new, previously unexplored dieback is being observed on Ash stands in the Mediterranean region. Given the importance of *Fraxinus* species in Mediterranean forest formations and the current, heavy impact of the disease, an investigation was carried out in this study in order to investigate the aetiology of the phenomenon. The study enabled the identification of some harmful fungal and oomycete pathogens as the etiological agents responsible for this widespread dieback.

Tree species of the genus *Fraxinus* play a crucial ecological role in Mediterranean forests. Though *Fraxinus* species are an important component of many mixed forests along the Italian Peninsula, *Fraxinus* decline has received until now limited attention. A progressive dieback of the three main species of *Fraxinus*, European ash (*Fraxinus excelsior* L.), flowering ash or manna ash (*Fraxinus ornus* L.), and narrow-leaved ash (*Fraxinus angustifolia* Vahl.), has been observed at various locations in central and north Italy (Panconesi et al., 2014; Benigno et al., 2023). Dieback of *Fraxinus* spp. was reported also in Switzerland (Rigling et al., 2018) and in other European countries, but in all those cases the symptomology was quite different and the damage was ascribed to the well-known pathogen *Hymenoscyphus fraxineus.* Symptoms exhibited by affected trees in the Mediterranean area included: sunken, ellipsoidal cankers along the stem and branches with bark cracks; leaf and shoot necroses (leading to a gradual decline of the canopy); exudate emission on the lower stem; root and collar rot; nonspecific canopy symptoms giving rise to a gradual or a sudden decline of trees (Orlikowski et al., 2011; Linaldeddu et al., 2020; Peters et al., 2023; Benigno et al., 2023). In some areas in particular (e.g. in the Colline Metallifere area, in Tuscany), damage was very high, with pronounced mortality, especially on young seedlings. The dieback strongly impacted natural regeneration.

Our investigation proved that in the Mediterranean region climatic anomalies, *in primis* heat and drought stresses occurring repeatedly during the growing season, weakened trees, strongly impairing their physiology and making them more prone to infection by thermophilic, opportunistic pathogens. Among the pathogens that benefited from such a climate pattern were

some members of the Botryosphaeriaceae family (Rehfeldt et al., 2009; Venette, 2009; Benigno et., 2023; 2024). The Botryosphaeriaceae (Botryosphaeriales, Ascomycetes) constitute a group of polyphagous phytopathogenic fungi, with a cosmopolitan distribution (Moricca et al., 2008, 2010; Linaldeddu et al., 2016; Moricca and Linaldeddu, 2017). Tree species affected by these group of pathogens normally show a variety of symptoms, among which cankers on the stem and branches, with distinctive necrotic wedge-shaped sectors in cross-section (Ragazzi et al., 1999; Linaldeddu et al., 2016; Manca et al., 2020). In many investigations on tree declines in which members of the Botryosphaeriaceae were involved, environmental stress turned out to be a key factor modifying the dynamics of host-pathogen interaction, favoring the microbial partner(s) of the antagonistic interaction (Sturrock et al., 2011; Hilario et al., 2019; Panno et al., 2021).

Over the years, several investigations tried to elucidate key aspects of the biology and epidemiology, including the endophytic nature of these fungi and their life history strategy. The taxonomy of the Botryosphaeriaceae was another important issue, as the taxonomic intricacy in this family has led to considerable confusion on their identity and thus also on the specific involvement of members of this group in the widespread declines that occurred worldwide (Dayarathne et al., 2016). Recent advances in molecular biology and diagnostic techniques have made available highly sensitive and effective tools that enabled a more accurate identification and characterization of the species of Botryosphaeriaceae. In the early 2000s, this group was divided into numerous distinct genera, all of which were previously unified under the teleomorph genus *Botryosphaeria* (Crous et al. 2006). The adaptability and global distribution of these pathogens underscore the urgent need for international management and control strategies (Úrbez-Torres, 2011).

Botryosphaeriaceae can infect plants through natural openings or wounds and most of them are endowed with an endophytic lifestyle (Slippers and Wingfield, 2007). During our survey, *Botryosphaeria dothidea* and *Diplodia fraxini* were isolated at a high frequency either from cankered tissues either from health plant material. Several studies have demonstrated that *B. dothidea* shows little or no host preference. This is confirmed by individual haplotypes occurring on multiple hosts, despite their surprisingly extensive distribution (Marsberg et al., 2017). The conidia and ascospores of *B. dothidea*, like thus of other Botryosphaeriaceae, are believed to be dispersed by wind and rain over relatively short distances (Ahimera et al., 2004; Amponsah et al., 2009; van Niekerk et al., 2010; Úrbez-Torres et al., 2010; Marsberg et al., 2017). *Diplodia fraxini*, although morphologically similar to *Diplodia mutila* (Fr.) Mont., is genetically distinct from it. Unlike what has been observed for *B. dothidea*, this fungus exhibit

a clear host specificity for the *Fraxinus* genus, including the ability to produce selective phytotoxic secondary metabolites (Cimmino et al., 2017; Benigno et al., 2023).

Decline phenomena are still more exacerbated by attacks of oomycetes of the *Phytophthora* genus. *Phytophthora* species pose a significant threat as emerging pathogens in various forested regions globally. They induce a wide range of symptoms in affected hosts, leading to the gradual or sudden decline of the canopy due to damage to roots an bark (Tyler et al., 2002). Forests have evolved in conjunction with a variety of organisms, including *Phytophthora* species, and generally, in the long run, these associations are not harmful to forest ecosystem. However, alterations in conditions, such as the introduction of pathogens into new environments, exposure to hosts without a coevolutionary history, or changes in the environment, can bring about shifts in the dynamics of forest pathogens, resulting in negative impacts on the ecosystem (Hansen, 2015).

Over the last two decades, numerous studies have demonstrated that several *Phytophthora* species are involved in the widespread decline of forest ecosystems in Europe (Jung et al., 2016). In our study too, the decline was in some instances associated to the action of *Phytophthora* species (Benigno et al., 2023). This introduces a further complication to investigations aimed at clarifying the etiology of *Fraxinus* decline, revealing that interactions in forests are often multi-partite and thus emphasizing the necessity of addressing multiple issues in the attempts to get to the bottom of the problem. The study carried out here is the most comprehensive survey of *Phytophthora* species affecting *Fraxinus* members, with a total of 11 different species identified. These include: *P. acerina*, *P. bilorbang*, *P. cinnamomi*, *P. hydropathica*, *P. lacustris*, *P. multivora*, *P. plurivora*, *P. polonica*, *P. pseudocryptogea*, *P. pseudosyringae*, and *P. syringae*. These taxa were reported for the first time as pathogens of species of the genus *Fraxinus* in Italy.

Current climatic trend seems to be particularly favorable for the lifestyle and development of this group of pathogens: increasingly mild and humid winters can induce an easy production of zoospores for host infection; this condition, combined with dry summers that cause severe stress in vegetation, makes the trees highly susceptible and easily attackable (Serrano et al., 2022).

Given the aforementioned considerations, and acknowledging the ecological importance of the species *Fraxinus ornus* in particular, it was considered appropriate to delve deeper into the causes of its decline/dieback. This deciduous tree is native to mixed hilly and mountainous forests in southern Europe and southwestern Asia. *F. ornus* is commonly used in conservation forests and for the re-colonization of degraded lands. Additionally, the production

of manna, a sweet-tasting sap used as a sweetener, enhances the economic value of this tree in southern Italy (Caudullo et al., 2016; Benigno et al., 2024).

The widespread observed mortality of flowering ash in affected regions proves the vulnerability of this species to pathogen attacks has been insufficiently studied thus far. This research represents in fact the first attempt to investigate the decline of *Fraxinus ornus* in Italy. One of the most evident symptoms observed on young *F. ornus* seedlings was the development of cankers on the stem. These cankers emerged repeatedly along the stem and caused a widespread mortality The death of the trees occurred when cankers, ellipsoidal in form and depressed over the bark, encircled the entire stem axis. Symptoms were also present on branches and shoots of young plants, with the shoots often bent on themselves. This latter symptom was mainly associated with the occurrence of *D. fraxini*.

All the symptoms observed on *Fraxinus* species are tangible indicators of tree decline and point to a combination of environmental and phytopathological stress factors (Benigno et al., 2023; 2024). As already mentioned, most studies on *Fraxinus* species focused until now on the damage caused by *H. fraxineus* (Fones et al., 2016; Mitchell et al., 2017; Madsen et al, 2021). In most investigations, *F. excelsior* seemed to be highly susceptible to *H. fraxineus* whereas *F. ornus* appeared less susceptible. This susceptibility classification aligns with a recent study by Carrari et al. (2015). Symptoms on woody parts caused by natural infections of *H. fraxineus* have not been observed in *F. ornus*, but necrotic lesions on the bark and wood discoloration on the stems have been documented (Kirisits et al., 2009; 2015; Papic et al., 2018). The hypothesis that *H. fraxineus* is confined to the leaves of *Fraxinus ornus*, without causing symptoms on woody parts, suggests a specific dynamic of interaction between the fungus and the tree (Schwanda and Kirisits, 2016). This characteristic could be a key element in understanding the relative resistance of *Fraxinus ornus* to ash dieback by *H. fraxineus*.

The results of our study indicate that fungi of the Botryosphaeriaceae family play a fundamental role in the decline of *F. ornus* in the Mediterranean area. Their presence in young *F. ornus* individuals emphasizes the need to deepen our understanding of their ecology and impact. Pathogenicity tests confirmed their pathogenicity on *F. ornus*. This demonstrates once again their role in the decline of this species and provides a solid foundation for understanding the specific dynamics of this pathological interaction (Batista et al., 2021). The impact of the disease has been particularly pronounced in nutrient-poor soils, on south or southwest-exposed slopes, and in formations subject to high temperatures and heatwaves, leading to significant mortality of young individuals, especially after prolonged periods of drought (Benigno et al., 2023). The higher mortality of young individuals in nutrient-poor soils underscores the

dependence of this tree species to nutrient availability, influencing its ability to resist pathogen attacks. Additionally, a higher incidence of the dieback in less dense woodland formations, in south or southwest-exposed slopes, suggests a close correlation with climatic conditions, such as exposure to direct sunlight and higher temperatures, conditions evidently favoring pathogen attacks and development. Forests subjected to high temperatures, heatwaves and drought are particularly vulnerable to infection by opportunistic pathogens, consistent with the broader context of climate change. In fact, the increased mortality of young *F. ornus* seedlings after prolonged drought indicates a critical connection between water stress and host susceptibility. Drought conditions weaken trees, making them more susceptible to infections by latent and opportunistic pathogens, promoting the emergence of endophytic fungi that can switch to a pathogenic lifestyle, what occurs precisely with the Botryosphaeriaceae (Desprez-Loustau et al., 2006; Benigno et al., 2023). The current climate of the Mediterranean region, characterized by rising temperatures, increasing water deficit, heatwaves, and prolonged drought, makes trees particularly vulnerable to attacks by these microbial entities.

A further complication when dealing with microorganisms endowed with an endophytic lifestyle is that their latent phase can cause them to be overlooked by quarantine measures (Slippers and Wingfield, 2007). The ability of these microbes to quickly cause diseases when their hosts are under stress makes them a significant threat to agricultural ecosystems, plantations, and native forests (Fones et al., 2016). This is especially relevant in conditions of dramatic climate change that increase stress on plant communities. In these conditions, it is crucial to maximize our understanding of the ecology and virulence of these pathogens, particularly as regards their endophytic nature, species richness, host-switching ability, and interaction with the host and the environment (Slippers and Wingfield, 2007).

An emerging, pathogenic endophyte which is currently causing problems to chestnut cultivation is the ascomycete *Gnomoniopsis castaneae* (Dennert et al., 2015; Maresi et al., 2013; Sakalidis et al., 2019). This pathogens causes the so called “brown or chalky rot” of nuts, a disease that strongly compromises fruit quality and organoleptic characteristics (Lione et al., 2019; Shuttleworth et al., 2013). The typical symptom is a brown discoloration of the endosperm, which appears dehydrated, spongy, or chalky with light brown, medium, and dark lesions along with discoloration (Shuttleworth et al., 2012; Maresi et al., 2013; Lione et al., 2015). The symptom is detectable only by removing the pericarp. Due to this feature, which is to be ascribed to the endophytic nature of the fungus, nut rot is observed mainly in post-harvest processing (Visentin et al., 2012; Maresi et al., 2013; Shuttleworth et al., 2013; Vettraino et al., 2019). Nuts may in fact appear healthy on the surface while the internal tissue is decayed

(Shuttleworth et al., 2013). This disease is considered responsible for millions of dollars of product losses (Shuttleworth et al., 2013).

G. castaneae has been recognized since the early 21st century as the primary agent of nut rot in chestnut groves across Europe and Oceania (Dennert et al., 2015; Shuttleworth et al., 2012; 2017; Visentin et al., 2012). The fungus was described almost contemporarily, in 2012, as *G. castaneae* in Italy (Visentin et al., 2012), and as *G. smithogilvyi* in Australia (Shuttleworth et al., 2012). These reports appeared in printed publications within weeks of each other but to date it is assumed that *G. castaneae* was the first to be published (Gonthier et al., 2017; Tamietti, 2016).

Implementing effective control measures against *G. castaneae*, primarily based on water treatment, sterilization, and post-harvest chemical applications, poses important challenges (Morales-Rodriguez et al., 2022; Silva-Campos et al., 2022; Bastianelli et al., 2022). In response to these challenges, we implemented a biological control method that focused on the application of competent strains of three *Trichoderma* species, namely *T. viride*, *T. harzianum*, and *T. atroviride*, by endotherapic treatments. Members of the genus *Trichoderma* have been reported as essential endophytes capable of interacting with various crop species (Zhang et al., 2016), providing a significant advantage to agriculture and preventing, at the same time, contamination of the environment by synthetic chemical residues.

Species of the genus *Trichoderma* are known to suppress plant pathogens by various mechanisms: direct parasitism, antibiosis, competition for nutrients and space (Harman et al., 2004; Ghazanfar et al., 2018; Zhang et al., 2022; Kim et al., 2023). Besides parasitising and restricting plant pathogens, *Trichoderma* species are also known to assist plants in overcoming environmental stresses and in realizing their yield potential by colonizing roots, therewith improving seedling vigor and the plant immune system (Prudencio et al., 2020; Agostini et al., 2023). In fact, *Trichoderma* spp. can act by increasing photosynthetic efficiency in stressed plants thus improving general plant growth performances (Rajesh et 2016; Kim et al., 2023). The mechanism through which *Trichoderma* induces biostimulation entails intricate communication at multiple levels with the root systems of the host plant, as elucidated by Brotman et al. (2013). Numerous strains of *Trichoderma* function as beneficial microorganisms, triggering the activation of defence and developmental responses in the host plant. Secondary metabolites, such as auxins, small peptides and other active compounds released into the rhizosphere by *Trichoderma* strains, have a fundamental role in promoting root branching and improving nutrient uptake capacity.

Pasche et al. (2016) hypothesized that the effectiveness of widely occurring endophytes such

as *Trichoderma atroviride* and *Bacillus amyloliquefaciens* as antagonists of *G. castaneae* in young seedlings substantiated in a deceleration of disease progression, although complete elimination was not achieved. They concluded that the total colonization of the plant by antagonists would lead to cessation of pathogen growth.

Up to date, endotherapy had never been employed to dispense Biological Control Agents (BCAs) in chestnut orchards. In our study, we embraced a biological control approach to reduce infections of the nut rot agent *G. castaneae* by administering *Trichoderma* species by endotherapic applications. This represented a completely new and unexplored strategy. Our treatments were highly successful as they efficiently restricted the growth and the incidence of *G. castaneae* in chestnut fruits. In the whole, the results obtained from both *in vivo* and *in vitro* tests, provided additional confirmation of the effectiveness of *Trichoderma* application as a biological control agent. In *in vitro* tests, *T. viride* and *T. harzianum* gave more promising results than *T. atroviride*, which exhibited the most unfavorable outcomes. However, the plant growth-promoting capabilities and biocontrol activity of *T. atroviride* have already been demonstrated (Rao et al., 2022). For these reasons, all the three *Trichoderma* species were employed in our endotherapic treatments, aiming to leverage the known capabilities of each one of these species.

To the best of our knowledge, no other author has reported a detailed assessment of the effects of the application of three antagonistic strains of *T. viride*, *T. harzianum*, and *T. atroviride* on *G. castaneae*. The use of *Trichoderma* as a biological control agent would provide a practical solution to specific phytosanitary challenges in chestnut forests. In fact, the exploitation of biological control of pathogens in forest environments could prevent environmental and health risks associated with chemical treatments, also because the use of chemical products in the forest is not permitted. Anyhow, the application of biological control methods against forest pathogens remains largely unexplored, with limited efforts directed toward mature trees.

Concluding remarks

The case studies examined in this thesis, along with numerous other compelling plant health issues in Mediterranean ecosystems, highlighted the emerging problem of the decline of forest formations and plantations, with climate change called into question as the major driver of the introduction of new pathogens into previously uncontaminated areas. The study also pointed out the inherent risk associated with global plant trade pathways that serve as inadvertent sources of unforeseen, unknown, and undesirable pathogens (Garbelotto and Pautasso, 2012; Panzavolta et al., 2021). To respond effectively to the threat of emerging pathogens, several countries are stepping up efforts to control the introduction of organisms harmful to agriculture and forestry. In an attempt to face the problem, plant health protection has been elevated to a key component of the agricultural and forestry policies of many countries. It is important to emphasize that careful consideration of disease prevention and mitigation issues is paramount in many forestry policies, e.g. in the development of assisted migration programs. In fact, in the struggle to conserve biodiversity amidst the growing challenges of climate change, the lack of an olistic, multidisciplinary approach, that takes into due consideration also the alarming problem of the introduction of microbial plant enemies, could paradoxically frustrate every effort, inadvertently leading to the loss of key species. International cooperation is also essential in developing preventive strategies to ensure that phytosanitary protocols are strictly implemented in plant trade processes, to prevent the introduction of harmful pathogens into new habitats. In this context, research plays a key role, not only for the development of sensitive and accurate diagnostic protocols, but also for the study of the biology and epidemiology of new pathogens, as well as for the assessment of the risk posed by the possible introduction of new pathogens into disease-free territories. This research also provided compelling evidence that the adoption of effective biological control approaches is a promising strategy to protect plant produce and mitigate the negative impacts on the environment and human health associated with the intensive use of fungicides.

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List of publication linked to the thesis

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