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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 been 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 ( $DI = n/N \times 100$ ) and the number of dead trees out of the total number of trees ( $M = d/N \times 100$ ), 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<sup>2</sup> 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<sup>2</sup>) 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<sup>-1</sup> 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	<i>Fraxinus angustifolia</i>			<i>Fraxinus excelsior</i>			<i>Fraxinus ornus</i>			Sites	Average temperature range(s) (°C)*	Average annual precipitation range(s) (mm)*
		R	TC	C	R	TC	C	R	TC	C			
<i>Armillaria mellea</i>	OR141502	-	-	-	-	3	-	-	-	-	1	13.7	986
<i>Botryosphaeria dothidea</i>	OR141137	-	-	-	-	-	4	-	-	20	1-20, 23, 35	4.0-15.1	887-1387
	OR119871												
<i>Diplodia fraxini</i>	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												
<i>D. mutila</i>	OR140929				-	-	5	-	-	-	21, 24	6.1-6.5	1250-2050
<i>D. seriata</i>	OR140930	-	-	1	-	-	7	-	-	7	2, 7-9, 13, 19, 20, 23, 34, 40	4.0-15.1	650-1387
	OR177959												
<i>D. subglobosa</i>	OR140931	-	-	-	-	-	18	-	-	5	3, 4, 6, 9, 20, 21, 24, 28, 29, 34	6.1-15.0	887-2050
	OR177962												
<i>Dothiorella iberica</i>	OR140932	-	-	-	-	-	-	-	-	1	34	14	1300
<i>Do. symphoricarposicola</i>	OR140933	-	-	-	-	-	1	-	-	-	20	6.5	1250
<i>Hymenoscyphus fraxineus</i>	OR140935	-	-	-	-	-	12	-	-	-	20, 23, 24, 31	4.0-7.6	1250-2050
<i>Neofusicoccum parvum</i>	OR140934	-	-	1	-	-	2	-	-	13	1-6, 8, 28, 37	13-15	887-1585
	OR177966												
<i>Phytophthora acerina</i>	OR140916	-	-	-	12	4	-	1	-	-	20, 22, 24, 28, 31, 33	6.1-13.0	1250-2050

(Continued)

TABLE 1 (Continued)

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

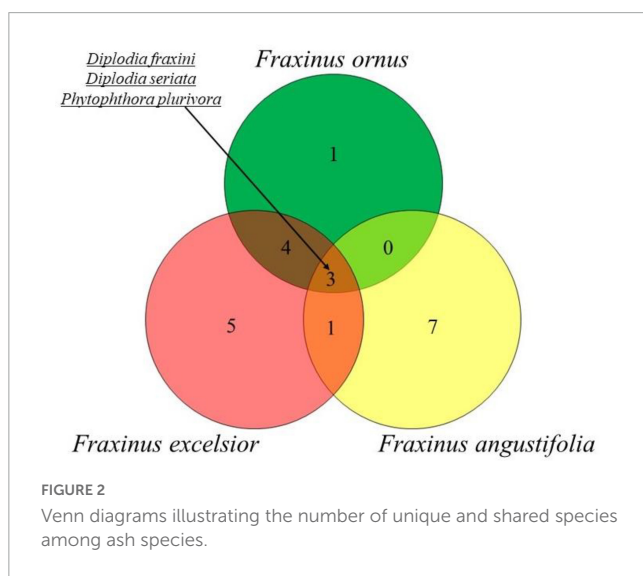
## 4. Discussion

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  $\pm$  standard deviation caused by each *Phytophthora* species inoculated on common ash seedlings.

Species	Isolate	Mean lesion length (cm)*	Re-isolation (%)
<i>P. acerina</i>	CB3	3.9 $\pm$ 1.1a	100
<i>P. cinnamomi</i>	CB121	2.8 $\pm$ 1b	100
<i>P. plurivora</i>	CB122	4.4 $\pm$ 1.7a	100
<i>P. pseudosyringae</i>	CB183	2.4 $\pm$ 0.4b	100
Control	-	0.5 $\pm$ 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.



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* (*Botryosphaerales*, 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 southwest, 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



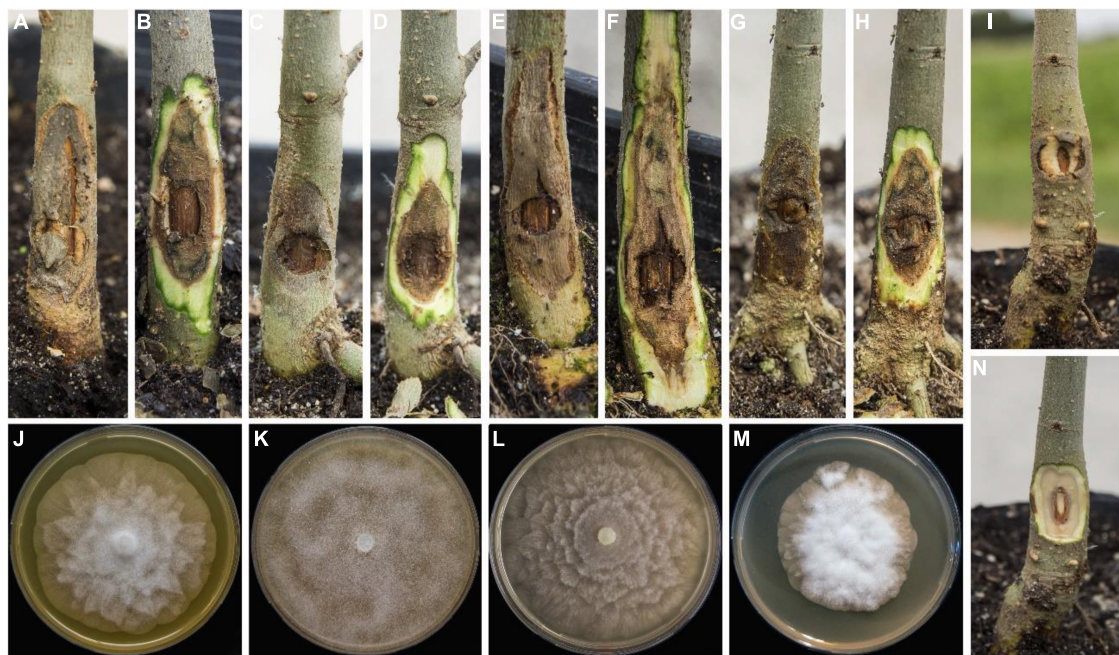


FIGURE 3

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>

- declining alder trees in Italy and description of *Phytophthora alpina* sp. nov. *Forests* 11:848. doi: 10.3390/f11080848
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