



Research article

Diversity and pathogenicity of Botryosphaeriaceae and *Phytophthora* species involved in emerging diseases of *Fagus sylvatica* in Italy and Slovenia

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Abstract

Extensive sunken and bleeding cankers, shoot blight, and root rot symptoms on European beech (*Fagus sylvatica* L.) trees have recently been observed in several forest ecosystems in Italy and Slovenia. Since there is little information about the aetiology of these diseases and given the high economic and ecological relevance of these formations, a study was conducted from summer to autumn 2023 in ten sites to define the occurrence, distribution and impact of the main pathogens involved. A total of 116 symptomatic samples were collected and processed. Based on colony appearance, morphological features and DNA sequence data, six species belonging to the genera *Botryosphaeria*, *Dothiorella*, *Diplodia*, *Neofusicoccum* (fungi) and *Phytophthora* (oomycetes) were isolated and identified. Pathogenicity tests performed on potted European beech seedlings satisfied Koch postulates and highlighted that *Diplodia corticola*, *Neofusicoccum parvum* and *Phytophthora plurivora* are the main agents involved in the extensive decline affecting European beech ecosystems in Italy and Slovenia.

Keywords

Diplodia corticola, beech decline, invasive species, emerging diseases, co-infections

Introduction

The European beech (*Fagus sylvatica* L.) is one of the most important and widespread broadleaved and deciduous trees in Europe, distributed from Sicily to southern Norway (De Rigo et al., 2016). Beech forests characterize the landscape of many mountain areas in Italy, from the Alps down to the southern regions of Campania, Basilicata, Calabria and Sicily in the Mediterranean area. Overall,



these formations cover over 1 million hectares (about 9% of the country's total forest area) (Nocentini, 2009). In Slovenia, beech formations occupy a major part of the forested area of the country and occur mainly in the montane and hilly zones, representing the most economically and ecologically important tree species with 33% in wood stock (Božič et al., 2010; Slovenian Forest Service, 2023).

Phytosanitary problems on European beech are historically related to endemic pathogens such as the beech cankers agent of the genus *Neonectria* Wollenw. and the charcoal canker agent *Biscogniauxia nummularia* (Bull.) Kuntze (Zabalgogezcoa et al., 2015). In addition, on older plants the settlement is common of fungi causing white rot, such as *Armillaria* spp., *Fomes fomentarius* (L.) Fr., *Inonotus hispidus* (Bull.) P. Karst. and *Ganoderma* spp. (Baum et al., 2003; Song et al., 2017; Gilmartin et al., 2022). Recently, new and previously unknown decline phenomena of beech forests have been impacting several regions of Europe; the symptoms are mainly linked to a progressive loss of foliage starting from the apical portions and cankers on the stem and branches. Several studies conducted in Europe showed a complex aetiology associated with these symptoms as well as a combined effect of extreme meteorological events and infections by *Phytophthora* species (Ruffner et al., 2019; Corcobado et al., 2020; Štraus et al., 2023; Tkaczyk et al., 2023). In particular, *P. cambivora* (Petri) Buisman and *P. plurivora* T. Jung & T.I. Burgess appear to be the two species most directly involved in the beech decline in Europe. However, many others have been reported, including the invasive pathogens *P. cinnamomi* Rands and *P. kernoviae* Brasier, Beales & S.A. Kirk (Jung et al., 2017; Corcobado et al., 2020). In Italy *P. cambivora*, *P. plurivora* and *P. pseudosyringae* T. Jung & Delatour were frequently isolated from declining beech from northern to southern regions (Bregant et al., 2023a; Riolo et al., 2022). In addition to *Phytophthora*, many other emerging pathogens have recently been isolated from declining beech in Europe (Langer and Bußkamp, 2021, 2023; Bencheva et al., 2023). In Germany after some exceptional drought seasons, strong decline phenomena of beech have been associated with the canopy attacks by different species of Botryosphaeriaceae, including *Botryosphaeria dothidea* (Moug.) Ces. & De Not. and *Diplodia corticola* A.J.L. Phillips, A. Alves & J. Luque (Langer & Bußkamp, 2023).

Given the alarming spread of decline and mortality phenomena in Italian and Slovenian beech forests and the limited information about the possible co-occurrence of attack by canopy and root pathogens, a thorough study was conducted to isolate, identify and characterize the main causal agents.

Materials and Methods

Field survey, isolation and characterization of the pathogens

Field surveys were conducted in ten beech forests distributed in North-Eastern Italy and Western Slovenia, ranging in altitude from 110 to 1600 m. a.s.l. (Table 1). Investigated beech formations were pure or mixed with oaks and sweet chestnut. During summer and autumn 2023, 50-meter linear transects were established at each site to estimate the disease incidence. All beech trees were visually inspected for the presence of disease symptoms (dieback, cankers on stem and branches, and root rot) and the tree mortality rate recorded. In order to clarify the causal agents of the main symptoms observed in the field, 34 representative trees were randomly chosen for sampling along the linear plots. Overall, a total of 87 samples were collected from stems and branches of 34 trees, including cankers and necrotic bark/wood lesions. In addition, a sample of rhizosphere and fine roots (300 g)

was collected at the collar of 29 plants, to investigate the occurrence of *Phytophthora* infections. All samples were labelled, stored at 4 °C and processed in the laboratory within 24-48 h.

Table 1 - Information on study sites and number of cankers (C) and rhizosphere (R) samples collected.

| Study sites | Country | Elevation (m a.s.l.) | Geographic coordinates | | Number of samples |
|-------------|----------|----------------------|------------------------|----------|-------------------|
| 1 | Italy | 270 | 45.31774 | 11.71689 | 2 (C), 1 (R) |
| 2 | Italy | 360 | 45.31827 | 11.70808 | 40 (C), 13 (R) |
| 3 | Italy | 240 | 45.29188 | 11.69684 | 5 (C), 2 (R) |
| 4 | Italy | 500 | 45.31226 | 11.68036 | 3 (C), 1 (S) |
| 5 | Italy | 1083 | 45.89385 | 11.63597 | 3 (C), 1 (R) |
| 6 | Italy | 1600 | 45.88058 | 11.39946 | 8 (C), 2 (R) |
| 7 | Italy | 1100 | 46.18464 | 13.41415 | 5 (C), 3 (R) |
| 8 | Slovenia | 110 | 45.94034 | 13.66677 | 7 (C), 4 (R) |
| 9 | Slovenia | 700 | 46.07093 | 12.57869 | 11 (C), 1 (R) |
| 10 | Slovenia | 1142 | 46.00949 | 13.83303 | 5 (C), 2 (R) |

In the laboratory, plant tissues were visually inspected, superficially disinfected with 70% ethanol for 30 s and the outer bark removed with a sterile scalpel. Longitudinal and transversal cuts were made to observe any internal symptoms. Isolations were made from approx. 3-5 mm² chips of inner bark and xylem cut aseptically from the margin of necrotic lesions (Linaldeddu et al., 2023). All chips were placed in 90 mm Petri dishes containing potato dextrose agar (PDA, Difco™, 39 g L⁻¹). After incubation at 20 ± 1 °C for 3–5 days in the dark, the growing fungal colonies were sub-cultured onto PDA and incubated at 20 °C in the dark. *Phytophthora* isolation from bark tissues and rhizosphere was performed as reported in Bregant et al. (2024a).

All colonies were initially grouped into morphotypes based on micromorphological features and colony growth patterns, including surface and reverse colony appearance observed after 7 days of incubation on PDA at 25 °C in the dark. Molecular analysis was applied to identify all isolates at the species level. The InstaGene Matrix (BioRad Laboratories, Hercules) was used to extract genomic DNA from the mycelium of 5-day-old colonies. The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified and sequenced using primers ITS1 and ITS4 (White et al., 1990). For the Botryosphaeriaceae species a portion of the translation elongation factor 1 alpha gene (*tef1-α*) was also amplified and sequenced with the primers EF446f and EF1035r (Inderbitzin et al., 2010). Polymerase chain reaction (PCR) mixtures and amplification conditions were as described by Linaldeddu et al. (2016, 2023). The PCR products were purified using a EUROGOLD gel extraction kit (EuroClone S.p.A.) following the manufacturer's instructions. The ITS regions were sequenced by the BMR Genomics DNA sequencing service (www.bmr-genomics.it), in both directions, with the primers used for amplification. The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza, Inc.; <http://www.geospiza.com/finchtv>) and then compared with reference sequences (type material) retrieved from the GenBank database using the BLASTn algorithm. ITS and *tef1-α* sequences from representative isolates obtained in this study were deposited in GenBank (Table 2).

Pathogenicity test

To verify the pathogenicity of the species investigated in this study, six isolates representing the four Botryosphaeriaceae and two *Phytophthora* species were artificially inoculated on three-year-old European beech seedlings in spring (April 2024). Six seedlings grown in plastic pots (10 cm diameter and 1.5 L volume) were inoculated with each of the six selected isolates, and six seedlings were used as controls. A total of 42 seedlings were used in the experiment. The isolates were inoculated on the stem. *Phytophthora* species were inoculated at the collar whereas Botryosphaeriaceae on the stem 10–15 cm above the ground. The inoculation point was initially surface-disinfected with 90% ethanol, and then a small wound (3 mm diameter) made with a sterile scalpel was inoculated with an agar-mycelium plug of the same size, taken from the margin of an actively growing colony on PDA. The inoculation site was covered with cotton wool soaked in sterile water and wrapped in a piece of aluminium foil secured with masking tape. Controls were inoculated with a sterile PDA plug applied as described above. All inoculated plants were kept in controlled conditions at 17–22 °C for 7 days and watered regularly. Re-isolation of inoculated species was attempted by transferring 10 pieces of inner bark and wood tissues taken from the margin of each lesion onto PDA and PDA+ (Linaldeddu et al., 2023). The stem was surface disinfected with 90% ethanol before re-isolation. The cultures obtained were grown in daylight at room temperature and then identified by morphological and molecular analysis (ITS region) as described above.

Statistical analysis

Data from the pathogenicity assay were first checked for normality, and then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined using the LSD Multiple Range test ($P = 0.05$) following a one-way ANOVA. Statistical analyses were performed using XLSTAT 2008 software (Addinsoft, France).

Results

Field survey

Field surveys conducted in ten European beech stands in Italy and Slovenia revealed the presence of severe diseases with a complex symptomatology and a strong impact on tree vitality. On stem and branches of the affected trees extensive sunken cankers characterized by inner bark necrosis and internal wood discoloration were consistently observed (Fig. 1). In the cross section, sunken cankers showed the typical V-shaped necrotic sector caused by Botryosphaeriaceae (Fig. 1). The lesions progressively covered the whole section causing death of the branches and the emission of epicormic shoots. On the outer bark surface around the cankers, pycnidia of Botryosphaeriaceae were very abundant (Fig. 1). In addition, affected trees showed leaf chlorosis, stunted growth, sudden-death symptoms, necrotic bleeding cankers and inner-bark lesions at the collar and main roots, as well as loss of fine roots (Fig. 1). The highest incidence of symptomatic trees was observed at sites 1, 2, 3, 8 and 9 (100% of monitored trees). The highest tree mortality rate was detected at sites 1, 2 and 8 (32%, 28% and 27%, respectively), while a significant number of visually asymptomatic trees (70%) was observed at site 6.

Aetiology

Isolations performed from 87 symptomatic branch samples yielded a total of 83 fungal colonies representing the genera *Botryosphaeria*, *Diplodia*, *Dothiorella* and *Neofusicoccum* (Table 2).

Table 2 – Sequences accession numbers deposited in GenBank and number of isolates of each species obtained in this study from cankers and rhizosphere.

| Fungal species | Accession number | | Number of isolates | | Number of sites |
|--------------------------------|------------------|---------------|--------------------|-------------|-----------------|
| | ITS | <i>tef1-α</i> | Cankers | Rhizosphere | |
| <i>Botryosphaeria dothidea</i> | PQ240108 | PQ246881 | 41 | - | 8 |
| <i>Diplodia corticola</i> | PQ240109 | PQ246882 | 28 | - | 5 |
| <i>Dothiorella omnivora</i> | PQ240110 | PQ246883 | 3 | - | 2 |
| <i>Neofusicoccum parvum</i> | PQ240111 | PQ246884 | 11 | - | 4 |
| <i>Phytophthora plurivora</i> | PQ240112 | - | 2 | 25 | 7 |
| <i>P. pseudosyringae</i> | PQ240113 | - | 3 | 4 | 2 |

On the basis of morphological features and DNA sequence data (ITS and *tef1-α*) four distinct species namely *B. dothidea* (41 isolates), *D. corticola* (28), *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (11) and *Dothiorella omnivora* Linaldeddu, Deidda & Scanu (3) were identified (Table 2). In addition, from 29 trees positive to Botryosphaeriaceae, two *Phytophthora* species were isolated from the rhizosphere and fine roots; these were *P. plurivora* (25 isolates) and *P. pseudosyringae* (4). *Phytophthora plurivora* and *P. pseudosyringae* were also obtained from 2 and 3 bleeding cankers on the stem, respectively (Table 2). Botryosphaeriaceae species showed a wide geographical distribution across the beech forests investigated in Italy and Slovenia. *Botryosphaeria dothidea* was the dominant species and was recorded in eight out of the ten sites and represented 49% of the total fungal isolates (Table 2, Figure 2). *Diplodia corticola* was the second most frequent species, isolated in five sites both in Italy and Slovenia.

Koch postulates

In the artificial inoculation trial, the selected isolates of *B. dothidea*, *D. corticola*, *D. omnivora*, *N. parvum*, *P. plurivora* and *P. pseudosyringae* proved to be pathogenic to European beech, although with different levels of aggressiveness (Tab. 3 and Fig. 2). *Phytophthora plurivora*, *N. parvum* and *D. corticola* were the most aggressive species. All species, except for *B. dothidea* and *D. omnivora*, caused wilting foliage on seedlings after seven days from the inoculation. All inoculated species were successfully re-isolated from necrotic stem tissues. Control seedlings inoculated with sterile PDA plugs remained symptomless.



Fig. 1 – Main disease symptoms observed on European beech trees in Italy and Slovenia: extensive canopy dieback (a-c); branches with active sunken cankers and inner bark discolorations (d-g); emission of epicormic shoots below the canker (h) cross-section of branches showing the V-shaped necrotic sector (i); abundant presence of pycnidia of *Botryosphaeria dothidea* and *Diplodia corticola* on the bark surface of the sunken cankers (j,k); bleeding cankers and dark wood necrosis caused by *Phytophthora* spp. (l-o); root rot (p).

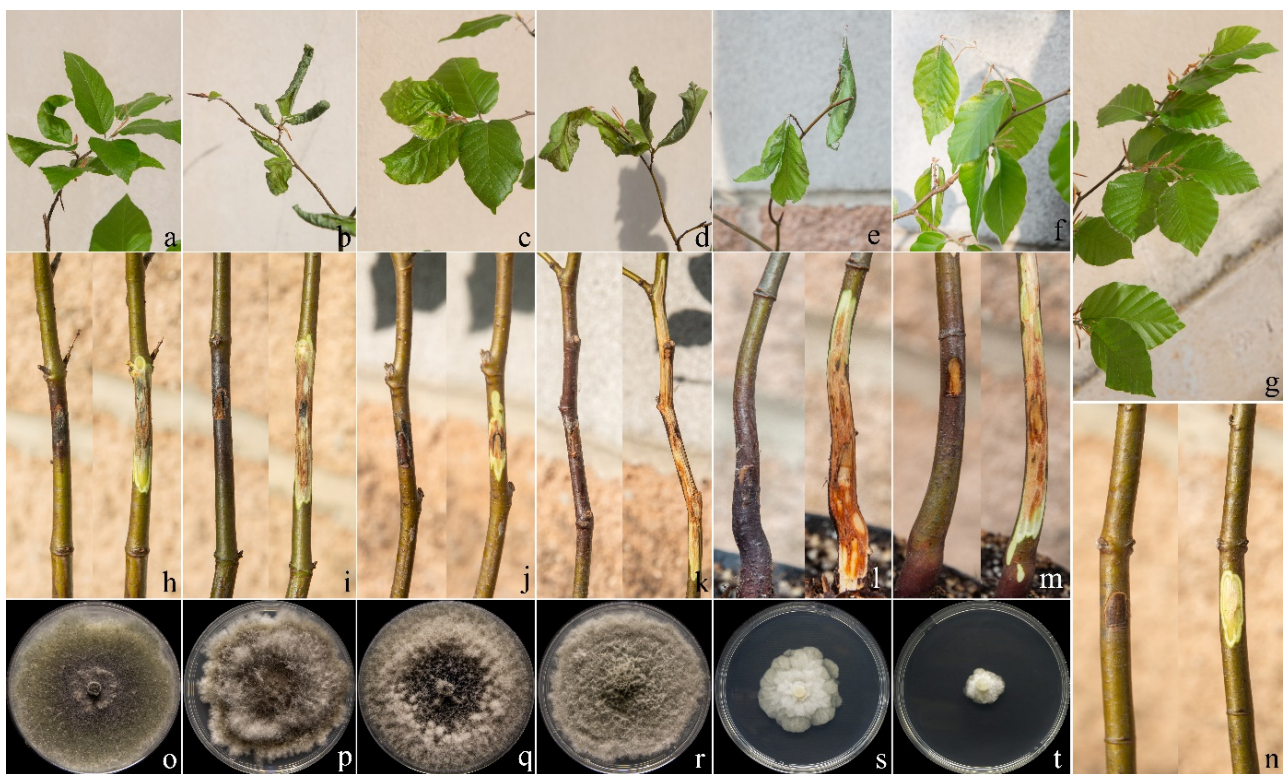


Fig. 2 – Foliar and wood symptoms observed on common beech seedlings seven days after inoculation with *Botryosphaeria dothidea* (a,h), *Diplodia corticola* (b,i), *Dothiorella omnivora* (c,j), *Neofusicoccum parvum* (d,k), *Phytophthora plurivora* (e,l) and *P. pseudosyringae* (f,m); Control seedling (g,n). Colony morphology of *B. dothidea* (o), *D. corticola* (p), *D. omnivora* (q), *N. parvum* (r), *P. plurivora* (s) and *P. pseudosyringae* (t) on PDA after seven days of incubation at 20 °C in the dark.

Table 3 – Mean lesion length (\pm standard deviation) caused by isolates of the six species on European beech seedlings and percentage of positive re-isolations.

| Species | Isolate code | Lesion length (mm) ^a | Wilted foliage | Re-isolation frequency (%) |
|--------------------------------|--------------|---------------------------------|----------------|----------------------------|
| <i>Botryosphaeria dothidea</i> | MR31 | 19,8 \pm 2,2c | no | 100 |
| <i>Diplodia corticola</i> | MR17 | 39,8 \pm 9,9b | yes | 100 |
| <i>Dothiorella omnivora</i> | MR19 | 11,2 \pm 2,1c | no | 100 |
| <i>Neofusicoccum parvum</i> | MR1 | 53,0 \pm 18,4a | yes | 100 |
| <i>Phytophthora plurivora</i> | MR2 | 62,2 \pm 5,9a | yes | 100 |
| <i>P. pseudosyringae</i> | MR7 | 35,5 \pm 6,3b | yes | 100 |
| Control | - | - | no | - |

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

Discussion and conclusions

During the past decades, several studies have investigated the possible role played by different fungi and oomycetes in the European beech decline aetiology in Europe, demonstrating that *Phytophthora* spp. linked to abiotic stresses are the main factors associated with the disease (Corcobado et al., 2020; Tkaczyk et al., 2023). Moreover, in a recent study conducted in Germany, many species belonging to the ascomycete family Botryosphaeriaceae have been found to be key pathogens involved in *Fagus sylvatica* decline, including *D. corticola* (Langer and Bußkamp, 2023).

This study confirms that Botryosphaeriaceae species represent the main component associated with branch canker and dieback symptoms, playing a primary role in the declining process. *B. dothidea*, *D. corticola* and *N. parvum* reported here for the first time as European beech pathogens in Italy and Slovenia were the most frequently isolated species from sunken cankers on stems and branches. Pathogenicity tests show that *N. parvum* and *D. corticola* are able to induce the same symptoms observed in nature, causing extensive sunken cankers and wilting symptoms after a few days. Species within the genera *Botryosphaeria*, *Neofusicoccum* and *Diplodia* have been reported as pathogens causing cankers, dieback and mortality on several woody hosts worldwide (Batista et al., 2021; Aiello et al., 2023).

Botryosphaeria dothidea is a latent pathogen of global importance to woody plant health, with more than twenty known host genera in crop and forest systems. In Europe it is reported on many native hosts such as *Ostrya carpinifolia* Scop. and *Fagus sylvatica* (Piškur et al., 2011; Marsberg et al., 2017; Langer and Bußkamp, 2023). *Neofusicoccum parvum* is an emerging species in temperate areas of the planet. Its rapid adaptation to new environments and hosts makes it a very dangerous and invasive pathogen (Batista et al., 2021). In Italy, this fungus is widespread across all 14 administrative regions, causing disease symptoms in a huge number of crop and forest systems (Aiello et al., 2023; Linaldeddu et al., 2023; Bregant et al., 2024b). The discovery of *N. parvum* in mountain areas of Northern Italy is of great scientific importance as it highlights how this species can also adapt to the cold climate of the Alps.

Diplodia corticola is a well-known pathogen of many *Quercus* species, it is naturally distributed with two different lineages across Mediterranean regions (Alves et al., 2004; Linaldeddu et al., 2013; Smahi et al., 2017). The geographic range of this species is rapidly expanding from the Mediterranean towards central Europe, probably helped by globalization and climate change (Langer and Bußkamp, 2023). It was recently reported in Germany on European beech and it appeared widespread on different *Quercus* species in Eastern United States such as Maine, Massachusetts, Tennessee and Wisconsin (Aćimović et al., 2016; Reed et al., 2018; Smith and Stanosz, 2018; Reyes et al., 2023). The discovery of *D. corticola* in Northern Italy and Slovenia beyond the 45th parallel and in submountain sites, confirms the expansion of this species towards northern and colder areas. Until some years ago, *D. corticola* was considered a primary pathogen restricted to plant species of the genus *Quercus* (Alves et al., 2004; Linaldeddu et al., 2014). It has only occasionally been isolated from different hosts such as grapevine (Úrbez-Torres et al., 2010). Recent studies have highlighted a rapid adaptation of this pathogen to other host species (host jump); in addition to European beech, it was found on ash, hornbeam, magnolia and laurel in Northern Italy (Bregant and Linaldeddu, unpublished). This could be the result of its expansion towards new geographical areas and the encounter with species different from those present in the Mediterranean climate. In consideration of the rapid spread of *D. corticola* to northern areas, a study is currently in progress to characterize the populations of *D. corticola* in Italy and Slovenia and understand its genetic diversity, distribution and host range.

The most interesting result of this study is the co-occurrence on the same tree of root pathogens such as *Phytophthora* spp. and canker causing agents belonging to the Botryosphaeriaceae family. *Phytophthora plurivora* has been isolated from almost all monitored trees, it is a species long known as one of the main pathogenic species of beech in Europe (Schoebel et al., 2014). *Phytophthora plurivora* emerged from roots and rhizosphere of symptomatic plants and less frequently from active

bleeding cankers in the lower part of the stem in seven sites of Italy and Slovenia. In addition to *P. plurivora*, in two cold sites *P. pseudosyringae* has been isolated, an emerging interesting pathogen well adapted to mountain habitats (Bregant et al., 2023a). It has already been reported on European beech in Italy, Slovenia, Germany, Spain and UK (Jung et al., 2003; Motta et al., 2003; Bregant et al., 2023a; Mullet et al., 2024). In the pathogenicity test, *P. plurivora* and *P. pseudosyringae* confirm their virulence, causing wilting symptoms of common beech seedlings after seven days. The co-association of Botryosphaeriaceae and *Phytophthora* infections on beech confirms what was recently found on other hosts such as ash, pomegranate, oak, olive and paulownia trees in Italy, showing the need to expand research on other crop and forest pathosystems (Linaldeddu et al., 2014, 2023; Benigno et al., 2023; Bregant et al., 2023b, 2024b). The onset of these increasingly frequent phenomena could also be linked to climate change, with greater plant susceptibility and adaptive capacity of these two groups of pathogens which are very different in evolutionary history but share some features such as wide host range and invasiveness (Benigno et al., 2023).

In conclusion, this study allowed us to expand knowledge on symptomatology and aetiology of European beech decline in Italy and Slovenia, revealing once again that both *Phytophthora* and Botryosphaeriaceae species represent a growing threat to forest ecosystems and agricultural crops (Bregant et al., 2023a; Linaldeddu et al., 2023). European beech decline is a complex disease caused by co-infections of multiple pathogens. Therefore, to adequately manage declining beech forests and protect still-undamaged forests, it is appropriate to take into due consideration the considerable diversity of pathogens involved.

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