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ORIGINAL ARTICLE

Characterization of chestnut bark fungal communities in healthy trees and blight recovered through natural or introduced hypovirulence

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Abstract
Various fungal species together with *Cryphonectria parasitica* (Murr.) Barr. have been isolated from chestnut tissues with blight symptoms. Microfungi remain in cankers during tissue healing, which occurs by transmitting hypovirulence by *Cryphonectria hypovirus* 1 (CHV1) compatible strains. However, studies focused on the diversity and ecology of the non-*C. parasitica* taxa on chestnut bark are lacking. This work evaluated the composition and richness of microfungi species associated with healthy chestnut trees, those with cankers healed by natural hypovirulence and those treated with hypovirulent strains (artificially introduced hypovirulence). Microfungi from diseased trees were isolated from six randomly selected points in the inner and external areas of the healed canker. In healthy trees, tissue samples were collected from 12 random locations on each tree's trunk. Fungal species were identified based on morphological characteristics and ITS region sequencing using the universal primers ITS1 and ITS4. Four hundred thirty-one fungal isolates were obtained from which 38 operational taxonomic units (OTUs) were identified. The fungal communities varied from tree to tree and did not display similar patterns. The endophyte *Biscogniauxia mediterranea* and epiphyte *Cytospora eucalypticola* fungi were detected in all study locations and tree health conditions. Notably, *C. parasitica* (virulent and hypovirulent strains) was dominant in the inner area of healed cankers, accounting for 64.3% of the isolates, and the saprobe fungi *Penicillium glabrum* was dominant among non-*C. parasitica* microfungi species. Dissimilarity analyses showed low similarity between the microfungi communities found in the inner and external areas of the healed cankers. The study reveals the long-life span of *C. parasitica* in healed cankers and the therapeutic effect of natural and introduced hypovirulence.

Keywords Healed cankers · Microfungal communities · *Cryphonectria parasitica* · *Biscogniauxia mediterranea* · *Pezicula ericae*

Introduction

Cryphonectria parasitica (Murr.) Barr., an A2 EPPO quarantine pathogenic fungus of Asian origin, was accidentally introduced to America and Europe through infected chestnut plants. In Europe, the disease was first reported in Northern Italy, in 1938, and caused great concern since the parasite showed a high aggressiveness in the European chestnut tree (*Castanea sativa* Mill) (Anagnostakis 1987; Rigling and Prospero 2018). The parasite progressively invaded neighboring countries, where it developed in an epidemic way, being present at the end of the 90s, in almost all European countries with chestnut stands (Robin and Heiniger, 2001), including Portugal (Abreu 1992). Notably, chestnut blight is present in all main chestnut-growing areas in continental Europe. This virulent fungus is a biotic factor that can infect and kill American (*Castanea dentata* (Marsh.) Borkh) and European (*Castanea sativa* Mill) chestnut trees. Infection is typically associated with the development of extensive necrosis (cankers) in the bark of the branches and trunk (Heiniger and Rigling 1994; Prospero et al. 2013). Cankers can rapidly increase, resulting in tree death (Prospero et al. 2013), causing severe environmental and economic damages.

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Hypovirulence is a natural phenomenon in which the aggressiveness of *C. parasitica* in the chestnut tree is reduced by the presence of *Cryphonectria hypovirus* 1 (CHV1), which promotes canker healing and chestnut recovery (Heiniger and Rigling 1994; Hoegger et al. 2003; Milgroom and Cortesi 2004; Robin et al. 2010). It has been proposed that CHV1 is introduced with its fungal host and naturally spreads throughout the entire area where *C. parasitica* is distributed (Bryner et al. 2012). However, in some areas of Europe where the presence of CHV1 has not been detected or its presence has been low, hypovirulence has been introduced following experimental programs defined by official entities (Zamora et al. 2014; Chira et al. 2017; Krstin et al. 2017; Diamandis 2018).

In chestnut bark infected by *C. parasitica*, the presence of other non-*C. parasitica* fungi has been reported (Robbins 1997; Akilli et al. 2009, 2011; Double et al. 2014; Kolp 2018; Kolp et al. 2020). It was also observed that the occurrence of other fungal species in cankers increases over time while *C. parasitica* decreases (Double et al. 2014). According to Kolp (2018), some fungal species, especially antagonists, may outcompete *C. parasitica*, reducing the pathogen's growth and, after some time, becoming the dominant fungi in cankers on surviving chestnut trees.

Biological control of chestnut blight by applying hypovirulent strains of *C. parasitica* results from the transmission of the hypovirus CHV1 by hyphal anastomosis between hypovirulent and virulent strains belonging to the same vc type (Grente 1965). After infection by CHV1, the virulent fungus weakens, and the treated canker stops expanding and becomes passive (Rigling and Prospero 2018). However, when the successful transmission of hypoviruses controls canker expansion, older portions of cankers can still harbor virulent *C. parasitica* (Jones 2008) since only actively growing mycelium is colonized by the hypovirus. Indeed, following hypovirulence transmission, the oldest areas of the dead bark may harbor virulent *C. parasitica* and fast-growing saprophytes (Double et al. 2014). In long-term assessments of treated cankers, Double et al. (2014) found that surviving trees exhibited diverse canker communities, often containing more non-*C. parasitica* overall than *C. parasitica* or hypovirulent *C. parasitica*.

The diversity and ecology of the non-*C. parasitica* taxa in the bark tissues of *C. sativa* are not very well known. Furthermore, studies are needed to evaluate microfungal community diversity and identify the most represented taxon within these communities and their biological behavior. A broader approach to the biodiversity of the microfungal biome will reveal interactions between *C. parasitica* and non-*C. parasitica* fungi, including endophytes and potential antagonists. In this sense, the objective of this work was to evaluate the composition of microfungi communities associated with chestnut bark in blighted trees that recovered by natural hypovirulence (NH) or introduced hypovirulence (IH) and compare the results with healthy trees (HT). This information may be beneficial to understand plant-microorganism interactions, and accurate knowledge of the composition of microorganisms on healed cankers may be essential to develop effective biological disease control strategies.

Materials and methods

Study sites and sample collection

For this study, three stands were selected in the North region of Portugal. One stand was located in Bragança (41º38'31'' N, 6º46'53''W; 887 m.a.s.l.) and the other in Valpaços (41º33'48'' N, 7º28'43'' W; 855 m.a.s.l.), both in the Trás-os-Montes region. In Valpaços, only healthy trees (HT) were sampled. Bragança is a field assay where the introduction of hypovirulent strains (IH) has occurred since 2015. The other stand was located in Felgueiras (Douro Litoral) (41º22'47'' N 8º10'56'' W; 369 m.a.s.l.), where a natural hypovirulence spread (NH) has occurred since 2013 (Ibáñez, 2015).

The Bragança stand has an area of 3 ha with approximately 20-year-old trees belonging to the cv. Longal and spaced 10 × 10 m apart. The Valpaços stand covers an area of 1 ha with trees of approximately 25 years old belonging to cv. Judia and spaced 10 × 10 m apart, and in Felgueiras stand covers about 1 hectare with hybrid plants (*Castanea sativa* × *Castanea crenata*) resistant to ink disease spaced 5 × 5 m apart. In Felgueiras, hybrid chestnut rootstocks resistant to ink disease were grafted mainly onto the Longal and Martaínha varieties. The Judia and Longal varieties are the most cultivated in the NE of Portugal, on Trás-os-Montes and the Martaínha variety is characteristic of Soutos da Lapa, in the districts of Viseu and Guarda (region of Beira Alta). Longal is the oldest variety, and is considered the best variety for the industry. The Judia and Martaínha varieties are usually preferred to sell fresh fruit due to their greater size (Costa et al. 2008).

Sampling was performed in Bragança (IH) and Valpaços (HT) in 20 selected chestnuts trees and 16 trees in Felgueiras (NH). The tissues were collected at six random points in the inner area of healed cankers and at six random points in the external area of each healed canker (a total of 12 samples in each tree) using a T Lok™ puncture biopsy needle (Jorgensen Laboratories, Inc.) to obtain microfungi. In healthy trees, tissue samples were taken from 12 randomly chosen locations on the trunk of each tree. The samples were identified and promptly processed.

Fungal isolation

In the laboratory, samples obtained from healed cankers (inner and external) and healthy trees were disinfected by immersion in 50% (v/v) ethanol for 3 min. The samples were then placed on absorbent paper and transferred to Petri dishes (90 mm in diameter) with PDA (Potato Dextrose Agar - Difco, 39 gr/L) culture medium. Plates were incubated at 25 ± 2ºC in the dark and were observed daily for mycelial growth. A single plug was transferred to new PDA plates to obtain pure isolates for subsequent identification. The pure cultures of each fungus were preserved in PDA, kept at 4ºC, and then deposited in the fungal collection of the Laboratory of Plant Protection at Instituto Politécnico de Bragança (Portugal).

Identification of fungal species

The identification of the different species of fungi was based on morphological characteristics (Dugan 2006) and amplification and sequencing of the ITS (ITS1, 5.8 S, ITS2), ribosomal DNA (rDNA) region using the ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3′) universal primers (White et al. 1990) and compared to sequences published in the GenBank databases, BLAST (https://blast.ncbi.nlm.nih.gov/). Amplification via polymerase chain reaction (PCR) of DNA was executed in a 20 µL reaction volume containing REDExtract-N-Amp™ PCR Reaction Mix. PCR was carried out following the thermal cycling program: 4 min initial denaturation at 94 ºC, followed by 40 cycles of 30 s denaturing at 94 ºC, 1 min of primer annealing at 50 ºC, 1 min of extension at 72 ºC, and a final extension at 72 ºC for 10 min. PCR products (6 µL per well) were separated by electrophoresis at 80 V for 50 min on 1.5% agarose gels that were pre-stained with GelRed® Nucleic Acid Gel Stain (Biotium, Inc) in 1X TAE buffer and viewed under ultraviolet light. Amplified region sequencing was performed by Stabvida Laboratories (Caparica, Portugal). Each species was taxonomically identified according to the MycoBank Database (www.mycobank.org).

Incidence of *C. parasitica* virulent and hypovirulent strains in healed cankers

Identification of virulent and hypovirulent strains of *C. parasitica* was based on the morphological characterization of the isolates. For detection of hypovirulent strains, the isolates were incubated at 25 ± 2 °C for 7 days in the dark, followed by 5 days of incubation at room temperature with diffuse light. *C. parasitica* isolates were described as white, intermediate or orange according to the criteria cited by Hogan and Griffin (2008). Isolates that remained white at the end of this period were considered *C. parasitica* hypovirulent strains or *C. parasitica* white isolates. Isolates having more than 70% orange pigmentation were described as virulent *C. parasitica* (or orange isolates), and isolates that showed a slight orange color (between 30 and 70% orange pigmentation) were considered intermediate-pigmented isolates. The hypovirus CHV1 was confirmed in some white isolates by RT-PCR.

Diversity and composition of microfungi communities

The relative abundance of each fungal taxon at each sampling site was calculated as the total number of isolates of a taxon divided by the total number of isolates of all taxa.

The microfungi community in each sampling site was evaluated at the level of their richness (number of species) and abundance (number of isolates of each species). The diversity of microfungi at each study site was calculated using the Shannon & Wiener diversity index (*H'*) using the following the equation: $H' = -\sum p_i \ln p_i$, where p_i is the frequency of *i* microfungi in each population.

The Simpson's Diversity Index (1/D) was calculated at the inner and external areas of the healed cankers. Simpson's index (D) was calculated following the equation: $D = \sum p_i^2$, where p_i is the frequency of *i* microfungi in each population. Species evenness at the inner and external area of the healed cankers was estimated by calculating the Shannon equitability index (*J*) as indicated in the following equation: $J = H'/\ln S$, where *H'* is the Shannon & Wiener diversity index and S is the number of species in the sample. Bray-Curtis distances were computed based on square root transformed abundance data using function *vegdist* of package vegan in R-environment (R Core Team 2021; RStudio team 2021).

Data analysis

Statistical analyses of microfungi abundance in different sampling sites and healed cankers (inner and external areas) were performed using a one-way analysis of variance (ANOVA) or Kruskal-Wallis test after verifying the normality assumption using normal quantile-quantile plots and Shappiro-Wilk test, and homoscedasticy assumption using Levene test. All statistical analyses were performed using the Statistica 12 software (StatSoft Inc., Tulsa, OK).

Results

Description of microfungi community

In this study 672 samples were collected, 240 in Bragança (IH), 192 in Felgueiras (NH) and 240 in Valpaços (HT). Concerning abundance, 431 fungal isolates were obtained from all surveyed stands, with 190 in Bragança (IH), 118 in Felgueiras (NH) and 123 in Valpaços (HT). Bragança (IH) had the highest average of number of isolates per tree (mean of 9.50 with standard deviation of 1.99) and Felgueiras (NH) had the lowest value (mean of 7.38 with standard deviation of 1.67). In Bragança (IH) and Valpaços (HT) 19 different taxa (each) were identified and 11 different taxa were found in Felgueiras. Regarding microfungi diversity indexes, Valpaços (HT) had the highest Simpson Diversity Index (1.06) followed by Bragança (IH) (0.73) and Felgueiras (NH) (0.44) (Table 1).

In Table 1, we observe that the total number of taxa is higher in the external area of the cankers. Despite the average number of taxa being higher in the external area no significant differences were detected between the inner and external areas of cankers (Felgueiras, $X^2_{kw} = 3.5$, $P = 0.059$; Bragança, $X^2_{kw} = 3.9$, $P = 0.048$).

Overall, the isolation of microfungi identified 38 operational taxonomic units (OTUs), 30 of which were identified up to the species level and eight to the genera (Table 2). Three isolates could not be identified because the sequencing of the PCR product failed. Many OTUs were represented by only one or two isolates (55.3% of the total OTUs). The OTUs described in this study belonged to the phylum Ascomycota and Mucoromycota. Ascomycota was the most represented phylum with 98.4% of isolates, and the Mucoromycota only accounted for 0.93%. The Class Sordariomycetes showed the highest percentage of OTUs (75.9% of the total OTUs), including 16 families: *Chaetomiaceae*, *Coniochaetaceae*, *Coryneaceae*, *Cytosporaceae*, *Diaporthaceae*, *Gnomoniaceae*, *Graphostromataceae*, *Hypoxylaceae*, *Lasiosphaeriaceae*, *Melanommataceae*, *Nectriaceae*, *Ophiocordycipitaceae*, *Sordariaceae*, *Sporocadaceae*, *Valsaceae* and *Xylariaceae*, followed by the Class Leotiomycetes with 10.0% of OTUs from two families: *Dermateaceae* and *Helotiaceae*. The most abundant families were *Valsaceae* (45.9% of the total isolates), *Graphostromataceae* (9.7%) and *Dermateaceae* (9.7%).

The genus *Cytospora* was represented by five species, *Cytospora cedri* Syd., P. Syd. & E.J. Butler, *Cytospora diatrypelloidea* G.C. Adams & M.J. Wingf., *Cytospora eucalypticola* Van der Westh., *Cytospora ribis* Ehrenb., and *Cytospora unilocularis* Thambug., Camporesi & K.D. Hyde. Among these species, *C. eucalypticola* represented 3.0% (of the total isolates), *C. diatrypelloidea* represented

Table 1 Abundance and diversity of fungal isolated from healed cankers in the study locations: Bragança (introduced hypovirulence), Felgueiras (natural hypovirulence) and Valpaços (healthy trees)

Parameters	Introduced hypovirulence			Natural hypovirulence			Healthy trees
Number of samples	240			192			240
Total nº of isolates	190			118			123
Average isolate per tree	9.50 ± 1.99			7.38 ± 1.67			7.69 ± 2.89
Total nº of taxa	19			11			19
Shannon-Wiener Index (H)	0.73			0.44			1.06
	Introduced hypovirulence			**Natural hypovirulence**			
	Inner area of canker	External area of cankers	External area of canker	Inner area of canker	External area of cankers		
Total nº of taxa	11	17	8	6			
Average nº of taxa	1.80 ± 0.77a	2.55 ± 1.32b	2.06 ± 0.93a	1.50 ± 0.63a			
Shannon-Wiener Index (H)	0.41	0.93	0.44	0.33			

2.8%, and *C. cedri* represented 2.3%. *C. parasitica* was the most represented species, with 198 isolates representing 45% of the total isolates.

Among non-*Cryphonectria parasitica* fungi isolates, *Biscogniauxia mediterranea* (De Not.) Kuntze (9.7% of the total isolates) was the most frequently isolated, followed by *Pezicula ericae* (Sigler) P.R. Johnst. (9.0%), *Penicillium* sp. (5.6%) and *Penicillium glabrum* (Wehmer) Westling (3.2%). *B. mediterranea* was the most frequent species in Bragança (IH) (21 isolates), Valpaços (12 isolates) and Felgueiras (NH) (nine isolates). *P. ericae* was the most frequent species in Valpaços (HT), with 38 identified isolates. This fungus was isolated from 10 trees (62.5% of total trees in Valpaços). *B. mediterranea* and *C. eucalypticola* were present in all situations, and *P. glabrum* was present in all healed cankers by natural or introduced hypovirulence.

The Venn diagram in Fig. 1 illustrates the relationships of OTUs across studied places and sanitary conditions. Only two OTUs are present in all studied situations, the endophytic *B. mediterranea* and the epiphyte fungi *Cytospora eucalypticola*. Few OTUs were shared between healthy trees (HT), introduced hypovirulent (IH) and natural hypovirulence (NH). Moreover, great dissimilarities are evidenced between the Trás-os-Montes region [Bragança (IH) and Valpaços (HT)] and Douro Litoral, Felgueiras (NH) stands.

Each OTU was also classified by its biological behavior (epiphyte/endophytes or saprotroph) by bibliographic research. Most OTUs on chestnut bark (18 out of 30) were classified as endophytes. Three OTUs were classified as epiphytes and the behavior of 16 OTUs is unknown. All OTUs were deposited in GenBank, and the accession numbers and percentage of nucleotide identity are presented in Supplementary Table 1.

Cryphonectria parasitica isolates

By visual observation of the morphological characteristics of the *C. parasitica* isolates, they were classified into virulent (white isolates) and hypovirulent (intermediate and orange isolates). Across all healed canker samples, approximately 64.3% of the isolates were *C. parasitica* (virulent and hypovirulent strains), and 35.7% were non-*C. parasitica* fungi. However, this percentage differed between locations. The percentage of *C. parasitica* and non-*C. parasitica* isolates obtained in Bragança (IH) Felgueiras (NH) and Valpaços (HT) are shown in Fig. 2A. The isolates of *C. parasitica* were higher than non-*C. parasitica* isolates in Bragança (IH) (56.3%) and in Felgueiras (NH) (77.1%). As expected, in Valpaços (HT), where no cankers exist, no isolates of *C. parasitica* were observed.

In total, 198 isolates of *C. parasitica* were recovered. In Bragança (IH) and Felgueiras (NH), 107 and 91 isolates of *C. parasitica* were recorded,. The isolates of *C. parasitica* were characterized as white isolates, intermediate isolates and orange isolates by the Hogan and Griffin (2008) criteria as described in the Material and Methods section (Fig. 2B). *C. parasitica* white isolates were dominant in Bragança (IH) (67.3%) while in Felgueiras (NH) *C. parasitica* orange isolates were dominant (68.1%). Intermediate-pigmented isolates accounted for 25.3% of *C. parasitica* isolates in Felgueiras (NH) and 14.0% of *C. parasitica* isolates in Bragança (IH).

Microfungi diversity in the inner and external areas of healed cankers

Significant differences were detected for the microfungi abundance in the different sampling sites (inner and external areas) in cankers with introduced hypovirulence (Bragança) ($X^2_{kw} = 8.4$, $P = 0.003$) and cankers with natural hypovirulence (Felgueiras) ($F = 13.89$, $P = 0.0008$). The microfungi abundance for Felgueiras (NH) was higher in the external area of healed cankers than in the inner area. In Bragança (IH), the microfungi abundance was higher in the inner area of healed cankers. Microfungi diversity varied between the sampling places (inner and external areas of healed cankers). Interestingly, in Bragança (IH) and Felgueiras (NH), and based on the Shannon-Wiener index, the inner area of healed cankers has less diversity than the external area (Table 3).

When comparing the two situations (i.e., introduced versus natural hypovirulence), it appears that the community of microfungi is less diverse in the cankers area where natural hypovirulence occurs. For example, in Felgueiras (NH), *C. parasitica* is dominant in the sampling area (inner and external areas), as indicated by Simpson's diversity index. Evenness varied between 0.18 and 0.20 in the healed cankers where there is a situation of natural hypovirulence and between 0.17 and 0.32 in the healed cankers treated by introduced hypovirulence. In both cases, there is no uniformity in the species distribution in the sampled cankers.

B. mediterranea was the most frequently isolated microfungi in the external area of healed cankers in both orchards (38.9% of total non-*C. parasitica* isolates in Felgueiras and 33.3% in Bragança) (Supplementary Fig. 1). In the inner area of healed cankers, the most frequently isolated species was *P. glabrum*, both in Felgueiras (44.4%) and Bragança (26.1%).

The dissimilarity values varied from 0.463 to 0.921 (Table 4). In Bragança (IH), there is approximately 50% similarity in the microfungi community between the inner and external areas of healed cankers. Moreover, the lowest similarity was found between the inner area of healed

Table 2 Cumulative number of fungi species isolated in Bragança (introduced hypovirulence) ($n=240$), Felgueiras (natural hypovirulence) ($n=192$) and Valpaços (healthy trees) ($n=240$)

Taxa (*Phylum*: *Class*: *Family*: *Species*)*	Bragança	Felgueiras	Valpaços	Total
Ascomycota				
Sordariomycetes				
Chaetomiaceae				
Arcopilus aureus	1	0	0	**1**
Coniochaetaceae				
Coniochaeta hoffmannii	0	0	1	**1**
Coniochaeta boothii	0	0	1	**1**
Coryneaceae				
Coryneum modonium	0	0	1	**1**
Cytosporaceae				
Cytospora cedri	7	0	3	**10**
Cytospora diatrypelloidea	6	0	6	**12**
Cytospora eucalypticola	1	1	11	**13**
Cytospora ribis	1	0	0	**1**
Cytospora unilocularis	3	0	0	**3**
Diaporthaceae				
Phomopsis sp.	0	1	0	**1**
Gnomoniaceae				
Gnomoniopsis castaneae	0	4	0	**4**
Graphostromataceae				
Biscogniauxia mediterranea	21	9	12	**42**
Hypoxylaceae				
Daldinia loculata	0	0	3	**3**
Lasiosphaeriaceae				
Fimetariella rabenhorstii	0	1	8	**9**
Fimetariella sp.	1	0	0	**1**
Melanommataceae				
Melanomma sanguinarium	0	0	4	**4**
Nectriaceae				
Fusarium solani	1	0	0	**1**
Fusarium sp.	1	0	0	**1**
Ophiocordycipitaceae				
Purpureocillium lilacinum	0	0	10	**10**
Sordariaceae				
Sordaria sp.	0	0	7	**7**
Sporocadaceae				
Pestalotiopsis cocculi	0	1	0	**1**
Valsaceae				
Cryphonectria parasitica	107	91	0	**198**
Xylariaceae				
Nemania serpens	0	2	0	**2**
Dothideomycetes				
Cladosporiaceae				
Cladosporium ramotenellum	2	0	0	**2**
Dothioraceae				
Aureobasidium pullulans	1	0	0	**1**
Montagnulaceae				
Paraconiothyrium brasiliense	0	0	5	**5**
Pleosporaceae				
Alternaria alternata	1	0	0	**1**
Phoma sp.	0	0	5	**5**
Eurotiomycetes				
Aspergillaceae				
Penicillium glabrum	10	4	0	**14**

Table 2 (continued)

Taxa (*Phylum*: *Class*: *Family*: *Species*)*	Bragança	Felgueiras	Valpaços	Total
Ascomycota				
Penicillium sp.	20	0	4	**24**
Leotiomycetes				
Dermateaceae				
Pezicula ericae	1	0	38	**39**
Pezicula sporulosa	0	2	0	**2**
Pezicula neocinnamomea	0	1	0	**1**
Helotiaceae				
Helotiales sp.	0	0	1	**1**
Pezizomycetes				
Pezizaceae				
Peziza varia	0	0	1	**1**
Plicaria endocarpoides	0	0	1	**1**
Mucoromycota				
Mucoromycetes				
Mucoraceae				
Mucor plumbeus	2	0	0	**2**
Rhizopus sp.	2	0	0	**2**
Others	1	1	1	**3**

n = number of samples, * According to Mycobank

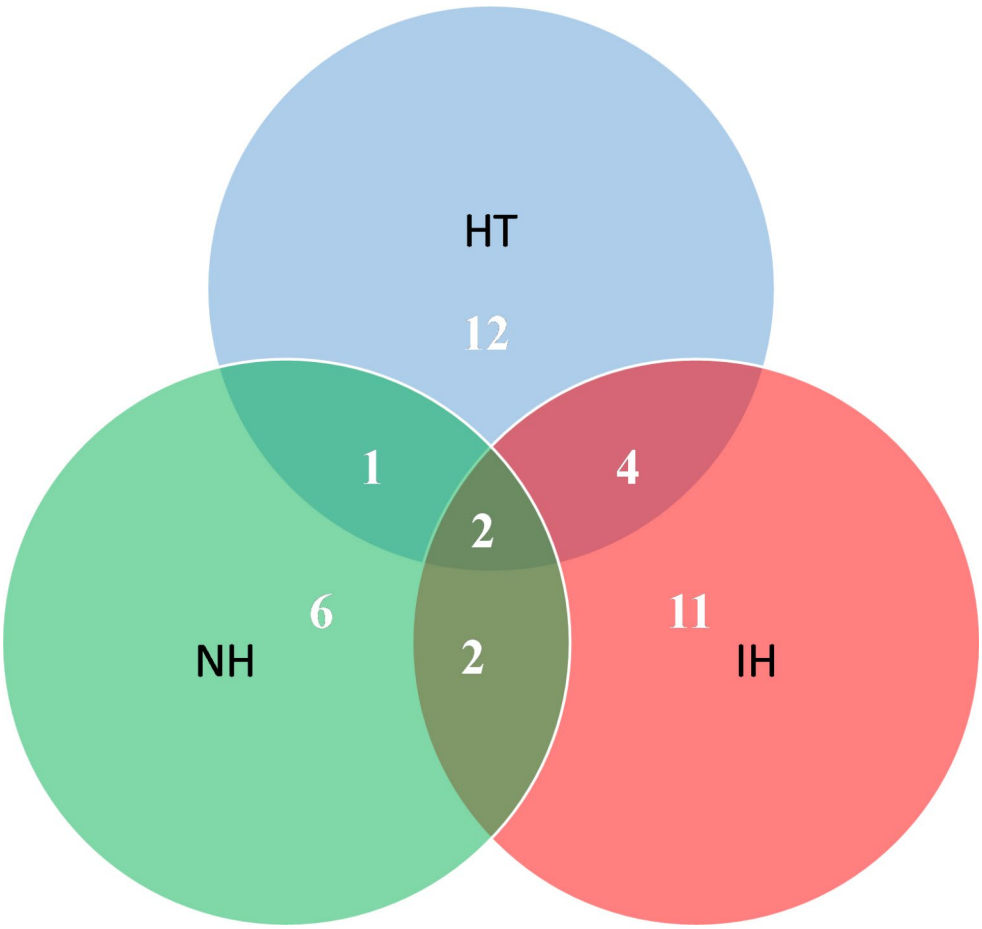

Fig. 1 Venn diagram depicting the distribution of Operational Taxonomic Units (HT – healthy trees, NH – natural hypovirulence, IH – introduced hypovirulence)

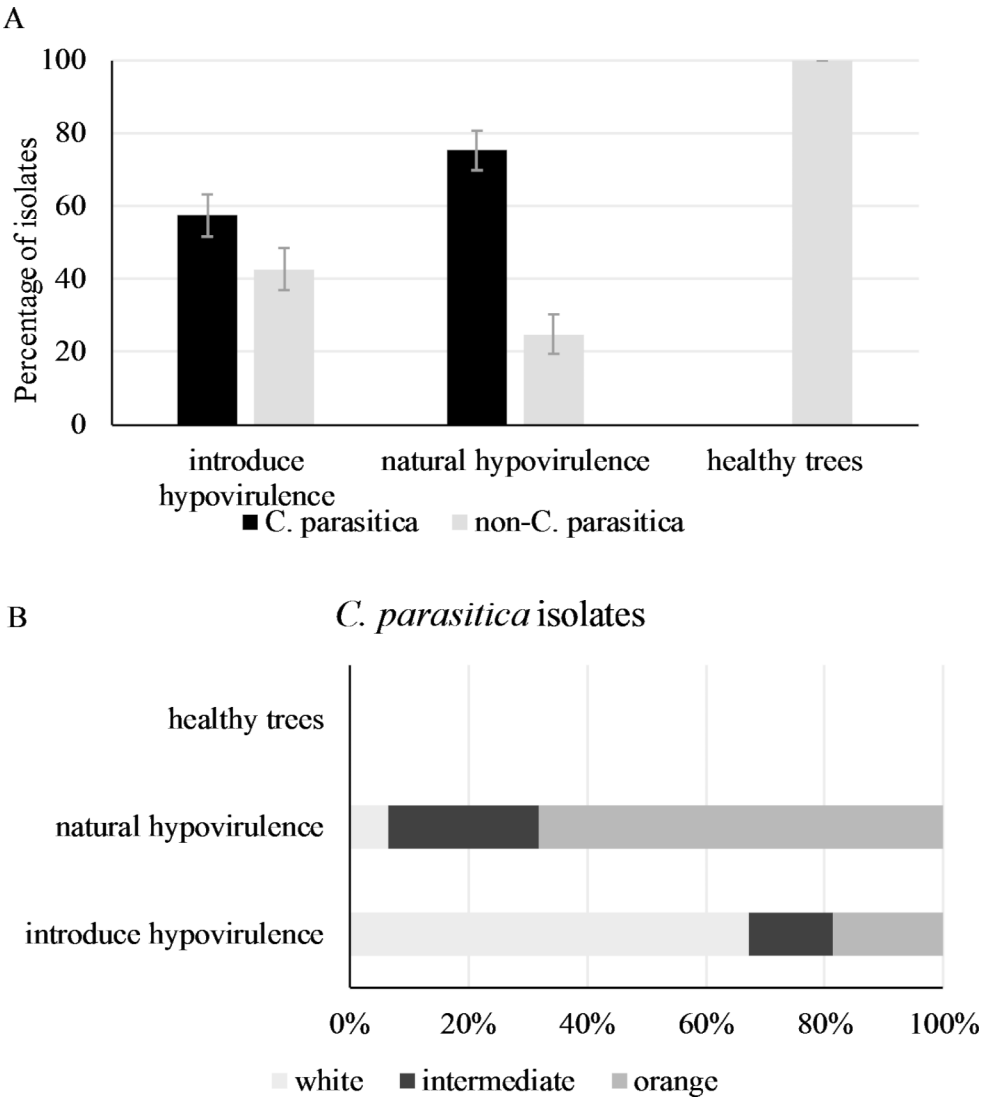

Fig. 2 A – Average percentage of *Cryphonectria parasitica* and non-*Cryphonectria parasitica* isolates in Bragança (introduced hypovirulence) ($n = 20$), Felgueiras (natural hypovirulence) ($n = 16$) and Valpaços (healthy trees) ($n = 20$), (vertical bars means standard error, n = number of trees). B – Percentage of *Cryphonectria parasitica* isolates (orange isolates – o, intermediate-pigmented isolates – i, white isolates – w) in Bragança (introduced hypovirulence), Felgueiras (natural hypovirulence) and Valpaços (healthy trees)

Table 3 Fungal isolates (Fi), Shannon & Wiener diversity index (*H'*), Simpson's diversity index (1/D) and Evenness (J) in the inner and external areas of healed cankers from Felgueiras (natural hypovirulence) and Bragança (introduced hypovirulence)

	Natural hypovirulence (Felgueiras)					Artificial hypovirulence (Bragança)				
	Fi	***H'***	**1/D**	**J**	**AIT**	**Fi**	***H'***	**1/D**	**J**	**AIT**
Inner area	47	0.33	1.51	0.18	2.94 ± 1.12a	109	0.41	1.59	0.17	5.45 ± 0.87a
External area	71	0.44	1.75	0.20	4.44 ± 1.15b	81	0.93	5.80	0.32	4.05 ± 1.32b

Fi – fungal isolates, *H'* – Shannon & Wiener diversity index, 1/D – Simpson's diversity index, J – Evenness. AIT – average of isolates per tree. Different letters in the column indicate significant differences

Table 4 Matrix of Bray and Curtis dissimilarity index values for microfungi community isolated from healed cankers in two sites (inner and external areas) in Bragança and Felgueiras. Axes are labeled with sampling sites

	Bragança IAC	Bragança EAC	Felgueiras IAC	Felgueiras EAC
Bragança IAC	0.000			
Bragança EAC	0.463	0.000		
Felgueiras IAC	0.708	0.794	0.000	
Felgueiras EAC	0.921	0.754	0.724	0.000

IAC – inner area of cankers, EAC – external area of cankers

cankers in Bragança (IH) and the external area of healed cankers in Felgueiras (NH) (0.921).

Discussion and conclusion

In this study, we described the microfungi communities associated with healed cankers in natural or introduced hypovirulence and the microfungi communities associated with healthy chestnut trees.

The abundance and diversity of microfungi vary between groves and sampling chestnut trees. The diversity was greater in healthy trees (Valpaços), and *C. parasitica* is the dominant species in groves with healed cankers by natural or introduced hypovirulence (Bragança and Felgueiras). When the sites with healed cankers (natural and introduced hypovirulence) are compared, the statistical analysis showed differences in the abundance of microfungi between the external and inner areas of healed cankers. The highest diversity was found in the inner area of healed cankers for introduced hypovirulence and the external area of the healed cankers for natural hypovirulence. This study showed a low similarity between sample areas (inner and external area) and between study sites (Bragança and Felgueiras). The greater distance between fungal communities observed between Bragança (Trás-os-Montes region) and Felgueiras (Douro Litoral region) may be associated with ecological and geographic conditions but also the genetic background of trees and applied practices to manage the hybrid (*C. crenata* × *C. sativa*) stand where natural hypovirulence was evidenced.

C. parasitica was the most frequently isolated species from healed cankers (by natural spread or introduced hypovirulence), and *P. ericae* was dominant in healthy trees. Natural dissemination and introduced hypovirulent strains have led to a high prevalence of hypovirulence in many areas in Europe (Heiniger and Rigling 1994). After infection by the hypovirus CHV1, the active canker eventually stops expanding and becomes passive (Rigling and Prospero 2018). According to Ježić et al. (2019), the callus tissue formation around a canker generally begins in the first growing season after acquiring a hypovirus by the virulent *C. parasitica* isolate but may take several years for significant callus tissue to develop. During this time or even several years after complete healing of the cankers, active mycelium from virulent and hypovirulent *C. parasitica* has been isolated (Bryner et al. 2014; Double et al. 2014; Ježić et al. 2019; Coelho et al. 2021).

The white culture morphology is generally an indicator of hypovirus infection (Grente 1978; Hogan and Griffin 2008). Since, the detection of CHV1 by molecular methods was not performed in our study, these isolates were considered putatively hypovirulent. In this study, the presence of *C. parasitica* hypovirulent strains varied with the sampling site. In Bragança (IH), where hypovirulence has been introduced since 2015, 67.3% of *C. parasitica* hypovirulent strains were observed, while in Felgueiras (NH) the number of *C. parasitica* white isolates was lower with only 6.6%. One of the reasons for the higher number of *C. parasitica* white isolates in Bragança could be due to the introduced application of the hypovirulent strains by scaring over the whole cankers area. Despite the introduced hypovirulence in Bragança, isolates obtained 5 years after the application showed 18.7% of the *C. parasitica* isolates with orange morphology. Jones (2008) also observed older portions of cankers that still harbor virulent *C. parasitica* even when the successful transmission of hypoviruses controls canker expansion.

In both locations, many *C. parasitica* intermediate-pigmented isolates were also present. The percentage of intermediate-pigmented isolates was higher in Felgueiras (NH) stand (25.3% of *C. parasitica* isolates), with natural hypovirulence. It was worth mentioning that others have reported the presence of intermediate-pigmented isolates from healed cankers (Coskun et al. 1999; Hogan and Griffin 2002, 2008). Additionally, Hogan and Griffin (2008) found that some intermediate-pigmented isolates tested positive for the presence of CHV1 and that isolates may perform an essential function in the biological control of chestnut blight.

P. ericae was the most abundant species in healthy trees (Valpaços), and one single isolate was recovered from healed cankers in Bragança. In a review on endophytic fungi in chestnut trees, Nicoletti et al. (2021) refer to the fungus *P. ericae* isolated from chestnut (*C. dentata*) branches with canker. Moreover, Ibañez (2015) isolated the endophyte fungi *Cryptosporiopsis ericae* Sigler, the anamorph form of *P. ericae*, from active cankers on chestnuts, and in laboratory studies found that the fungus had no antagonistic activity against *C. parasitica*.

B. mediterranea was the most representative species in Bragança (IH) and Felgueiras (NH), and it was the second most frequent species in healthy trees (9.8% of the total isolates). *B. mediterranea* is a fungus frequently associated with active or healed cankers in northern Portugal (Gouveia et al. 2017; Coelho et al. 2021). The fungus *B. mediterranea* was first detected in Portugal in 1930 and has been associated with several forest species' early stages of decay. This species has a strong incidence on *Quercus* species, including the cork oak, and has also been isolated from chestnut trees (Henriques et al. 2015). This fungus can live as an endophyte in the plant and act as an opportunistic parasite when trees show symptoms of decline (Henriques et al. 2014).

Cytospora was the most represented genus, with 9.0% of total isolates recovered in this study. *Cytospora* species are fungi that cause most common cankers, and tree decline (Fotouhifar et al. 2010), and some species of this genus are described as pathogenic to chestnut trees (Suarez 1989; Dar and Rai 2014; Jiang et al. 2020). In recent studies performed in the north of Portugal, *C. eucalypticola* was found in active (Ibañez 2015) and healed cankers (Coelho et al. 2021)d *diatrypelloidea* was reported to be one of the most abundant species recovered from healed cankers (Coelho et al. 2021).

For the other *Cytospora* species detected in this study, there are no references to these species in chestnut trees.

Penicillium was also largely represented in this study. For example, in Bragança (IH), *Penicillium* sp. and *P. glabrum* were abundant in this stand. The genus *Penicillium* is a cosmopolitan fungus, present in various environments and composed of about 300 species (Thom 1930). This fungus is prevalent in chestnut nuts (Rodrigues et al. 2013) and is one of the primary contaminants in post-harvest conditions. Double et al. (2014) reported a large number of isolates of *Penicillium* spp., including the species *P. glabrum* isolated from hypovirulent cankers in American chestnut. Additionally, studies in the north of Portugal (Coelho et al. 2021) confirmed the presence of *Penicillium* in healed cankers on European chestnut trees.

Purpureocillium lilacinum (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson represented 2.32% of the total species (10 isolates). *P. lilacinum* is a species of filamentous fungus in the family Ophiocordycipitaceae (Spatafora et al. 2015), and it has been isolated from a wide range of habitats and has potential as a biocontrol agent. This species is considered a parasitic fungus on eggs, female adults, and larvae of insects and nematodes (Schapovaloff et al. 2014; Barra et al. 2015). In recent laboratory studies, Toksöz et al. (2018) showed virulence of *P. lilacinum* against adults of ambrosia beetles (*Xylosandrus germanus* and *Xyleborus dispar*), pests of many forest species including chestnut trees.

Other species were underrepresented in the present study. Among these species, endophyte species, including *Gnomoniopsis castaneae* Tamietti and *Arcopilus* (= *Chaetomium) aureum* Chivers stand out. *G. castaneae* an emerging pathogen in chestnut cultivation worldwide, can exist on chestnut trees as an endophyte without causing any symptoms (Pasche et al. 2016; Lione et al. 2019; Dobry and Campbell 2022). This species was found only in Felgueiras (NH) with four isolates (representing 0.9% of the total isolates). *Arcopilus. aureum* had only one isolate in Bragança (IH). Many species of the genus *Chaetomium* are described as antagonists of plant fungi, and the secondary metabolites of these species exhibit antifungal activity (Kabbaj et al. 2015). Recently, in vitro studies have shown that *A. aureum* can inhibit the mycelial growth of virulent *C. parasitica* by 37% (Coelho et al. 2022).

Endophytes represent a vital group of the fungal communities identified, with some species, such as *B. mediterranea*, *P. ericae* and *Penicillium* sp., highly represented in terms of abundance. It was also observed that some of them can turn pathogenic under unfavorable environmental conditions (Lo Presti et al. 2015; De Silva et al. 2019), which may compromise their potential as biocontrol agents. For example, *B. mediterranea* was reported as an endophyte in oak plants that acts as an opportunistic pathogen when the hosts suffer prolonged periods of stress (Henriques et al. 2014). *P. ericae* was the most abundant species in healthy trees. *Pezicula* are fungal genera containing tree pathogens within the Fagaceae (Kolp et al. 2020). Some species of this genus, including *Pezicula neocinnamomea* Chen Chen, Verkley & Crous or *Pezicula sporulosa* Verkley (Chandelier et al. 2019), are known to be endophytes of bark or stems of chestnut trees. In comparison, *C. ericae*, the anamorphic form of the *P. ericae*, was isolated from diseased chestnut trees (Ibañez 2015). Some species of *Penicillium* are typical saprotrophs on generic substrates and were previously recorded in different geographic areas and hosts (Farr and Rossman 2019).

The productivity of the chestnut tree is influenced by the multiple interactions established with different types of microorganisms, with the microfungi being the primary group of microbes associated with chestnut fitness (Nicoletti et al. 2021). Endophytic microorganisms are recognized as a promising group in terms of diversity and pharmaceutical potential (Wagenaar and Clardy 2001). Endophytes grow inter- or intracellularly, systemically or locally within their hosts without causing visible manifestations of infection or disease (Clay et al. 2016; Solis et al. 2016) and are thus considered potential candidates for biocontrol applications (Yue et al. 2000; Ek-Ramos et al. 2013; Oono et al. 2015; Potshangbam et al. 2017). Notably, in the present study, a high number (14 out of 38) of endophytic fungi co-occur with *C. parasitica* on healed cankers. *B. mediterranea* was more abundant in the external area of healed cankers in Bragança (IH) and Felgueiras (NH), and *P. glabrum* was more abundant in the inner area of healed cankers. The reason why *P. glabrum* was found in great abundance in the healed cankers' inner area may be due to its saprophytic behavior.

We characterized the chestnut bark fungi communities from three distinct regions of Portugal: Valpaços (HT), Bragança (IH) and Felgueiras (NH). Richness and composition of the fungal were analyzed in each stand following a systematic sampling approach distinguishing between healthy trees in Valpaços (HT) and recovered blighted trees with healed cankers (inner and external areas) by introduced or natural spread or hypovirulence in Bragança (IH) and Felgueiras (NH), respectively. The diversity in healthy trees was greater than in trees that recovered from blight, and the genus *Cytospora* was the most represented, with five species identified. Among the 38 OTUs, only *B. mediteranea* and *C. eucalyptica* are shared between all studied places and sanitary conditions. Richness is lower on the Douro Litoral stand, where a natural and massive spread of natural hypovirulence occurred. On the flip side, *C. parasitica* (virulent and hypovirulent strains) are dominant in healed cankers, and greater dissimilarity is found between the inner

and external areas. The external area was similar to healthy trees, which explains its greater fungal diversity. Due to the great abundance of *C. parasitica* isolates (virulent and hypovirulent strains), this study revealed the long-life span of *C. parasitica* (virulent and hypovirulent strains) in healed cankers and the therapeutic effect of natural or introduced hypovirulence.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Compliance with ethical standards This article does not contain any studies with human or animal subjects.

Conflict of interest The authors declare that they have no conflicts of interest.

References

Abreu CG (1992) A hipovirulência como forma de luta natural contra o cancro do castanheiro. Revista das Ciências Agrárias 15:167–169

Akilli S, Katircioðlu YZ, Maden S (2009) Chestnut blight cankers in black sea region of Turkey. Acta Hortic 815:247–252. https://doi.org/10.17660/ActaHortic.2009.815.32

Akilli S, Katircioðlu YZ, Maden S (2011) Biological control of chestnut canker, caused by *Cryphonectria parasitica*, by antagonistic organisms and hypovirulent isolates. Turk J Agric For 35:515–523. https://doi.org/10.3906/tar-0912-579

Anagnostakis SL (1987) America Chestnut Blight: the classical problem of an Introduced Pathogen. Mycologia 79:23–37. https://doi.org/10.2307/3807741

Barra P, Etcheverry M, Nesci A (2015) Improvement of the insecticidal capacity of two *purpureocillium lilacinum* strains against *Tribolium confusum*. Insects 6:206–223. https://doi.org/10.3390/insects6010206

Bryner SF, Rigling D, Brunner PC (2012) Invasion history and demographic pattern of *Cryphonectria hypovirus* 1 across european populations of the chestnut blight fungus. Ecol Evol 2:32927–33241. https://doi.org/10.1002/ece3.429

Bryner SF, Prospero S, Rigling D (2014) Dynamics of *Cryphonectria hypovirus* infection in Chestnut Blight Cankers. Biol Control 104:918–925. https://doi.org/10.1094/PHYTO-03-13-0069-R

Chandelier A, Massot M, Fabreguettes O, Gisher F, Teng F, Robin C (2019) Early detection of *Cryphonectria parasitica* by real-time PCR. Eur J Plant Pathol 153:29–46. https://doi.org/10.1007/s10658-018-1538-0

Chira D, Bolea V, Chira F et al (2017) Biological Control of *Cryphonectria parasitica* in Romanian Protected Sweet Chestnut forests. Not Bot Horti Agrobo Cluj-Na 45:632–638. https://doi.org/10.15835/nbha45210895

Clay K, Shearin ZRC, Bourke KA, Bickford WA, Kowalski KP (2016) Diversity of fungal endophytes in nonnative *Phragmites australis* in the Great Lakes. Biol Invasions 18:2703–2716. https://doi.org/10.1007/s10530-016-1137-y

Coelho V, Nunes L, Gouveia E (2021) Short and long term efficacy and prevalence of *Cryphonectria parasitica* Hypovirulent strains released as Biocontrol Agents of Chestnut Blight. Eur J Plant Pathol 159:769–781. https://doi.org/10.1007/s10658-021-02200-3

Coelho V, Machado J, Guerra I, Gouveia E (2022) In vitro antagonism evaluation of endophyte Chaetomium aureum against Cryphonectria parasitica. ISPEC 9th International Conference on Agriculture Animal Sciences and Rural Development 19–20 March Burdur Turkey Oral presentation Abstract book ISBN: 978–625–8405–08–8:131

Coskun H, Turchetti T, Maresi H, Santagada A (1999) Preliminary investigations into Cryphonectria parasitica (Murr.) Barr isolates from Turkey. Phytopathol Mediterr 38:101–110

Costa R, Ribeiro C, Valdiviesso T, Afonso S, Borges O, Soeiro J, Costa H, Fonseca L, Augusta C, Cruz MH, Salazar M, Matos Soares F, Sequeira J, Assuncão A, Correia P, Lima MJ (2008) Variedades de Castanha das Regiões Centro e Norte de Portugal., ISBN 978-989-95658-2-1, Ed. INRB.I.P., 80 pp

Dar MA, Rai MK (2014) Occurrence of *Cytospora castanae* sp. nov., associated with perennial cankers of *Castanea sativa*. Mycosphere 5:747–757. https://doi.org/10.5943/mycosphere/5/6/5

De Silva NI, Brooks S, Lumyoung S, Hyde KD (2019) Use of endophytes as biocontrol agents. Fungal Biol Rev 33:133–148. https://doi.org/10.1016/j.fbr.2018.10.001

Diamandis S (2018) Management of Chestnut blight in Greece using hypovirulence and silvicultural interventions. Forests 9:492. https://doi.org/10.3390/f9080492

Dobry E, Campbell M (2022) *Gnomoniopsis castaneae*: an emerging plant pathogen and global threat to chestnut systems. Plant Pathol 72:218–231. https://doi.org/10.1111/ppa.13670

Double ML, Kolp MR, Jarosz AM, Davelos Baines A, Fulbright DW, MacDonald WL (2014) Fungi associated with hypovirulent cankers of differing ages on american chestnut. Acta Hortic 1043:57–65. https://doi.org/10.17660/ActaHortic.2014.1043.6

Dugan FM (2006) The identification of Fungi: an Illustrated introduction with Keys, Glossary and Guide to Literature. The American Phytopathological Society, St. Paul, MN USA

Ek-Ramos MJ, Zhou W, Valencia CU, Antwi JB, Kalns LL, Morgan GD, Kerns DL, Sword G (2013) Spatial and temporal variation in fungal endophyte communities isolated from cultivated cotton (*Gossypium hirsutum*). PLoS ONE 8:e66049. https://doi.org/10.1371/journal.pone.0066049

Farr D, Rossman A (2019) Fungal Databases, US National Fungus Collections, ARS. USDA. https://nt.ars-grin.gov/fungaldatabases. Accessed 20 April 2022

Fotouhifar KB, Hedjaroude GA, Leuchtmann A (2010) ITS rDNA phylogeny of iranian strains of Cytospora and associated teleomorphs. Mycologia 102:1369–1382. https://doi.org/10.3852/10-034

Gouveia E, Torrent II, Coelho V (2017) Diversidade de microfungos presentes nos ramos e troncos de castanheiro infectados por *Cryphonectria parasitica*. 8º Congresso Florestal Nacional. Viana do Castelo, 11–14 October, p. 193

Grente J (1965) Les Forms Hypovirulentes D'*Endothia Parasitica* it Les Espoirs De Lute Contre Le Chancre Du Chataignier, (C. R. Hebd). Seances Academie Agriculture France 51:1033–1036

Grente J (1978) L'utilisation de l'hypovirulence contagieuse – la lutte biologique contre le chancre a endothia du châtaignier. I.N.R.A. – Station de Pathologie Végétale Clermont – Ferrand: pp. 505–511

Heiniger U, Rigling D (1994) Biological control of chestnut blight in Europe. Annu Rev Phytopathol 32:581–599. https://doi.org/10.1146/annurev.py.32.090194.003053

Henriques J, Nóbrega F, Sousa E, Lima A (2014) Diversity of *Biscogniauxia mediterranea* within single stromata on Cork Oak. J Mycol 2014:ID324349. https://doi.org/10.1155/2014/324349

Henriques J, Nóbrega F, Sousa E, Lima A (2015) Morphological and genetic diversity of *Biscogniauxia mediterranea* associated to *Quercus suber* in the Mediterranean Basin. Revista de Ciências Agrárias 38:166–175

Hoegger PJ, Heiniger U, Holdenrieder O, Rigling D (2003) Differential transfer and dissemination of hypovirus and nuclear and mitochondrial genomes of a hypovirus-infected *Cryphonectria parasitica* strain after introduction into a natural population. Appl Environ Microbiol 69:3767–3771. https://doi.org/10.1128/AEM.69.7.3767-3771.2003

Hogan EP, Griffin GJ (2002) Spread of *Cryphonectria hypovirus* 1 into 45 vegetative compatibility types of *Cryphonectria parasitica* on grafted american chestnut trees. For Pathol 32:73–85. https://doi.org/10.1046/j.1439-0329.2002.00272.x

Hogan EP, Griffin GJ (2008) Importance of *Cryphonectria parasitica* stromata production and intermediate-pigmented isolates in spread of *Cryphonectria hypovirus* 1 on grafted american chestnut trees. For Pathol 38:302–313. https://doi.org/10.1111/j.1439-0329.2007.00537.x

Ibañez IT (2015) Hipovirulencia en *Cryphonectria parasitica*: dispersión natural e interacción interespecífica con hongos endofíticos e epifíticos. Dissertation. Instituto Politécnico de Bragança

Ježić M, Kolp M, Prospero S et al (2019) Diversity of *Cryphonectria parasitica* in callused chestnut blight cankers on european and american chestnut. For Pathol 49:e12566. https://doi.org/10.1111/efp.12566

Jiang N, Yang Q, Fan X-L, Tian C-M (2020) Identification of six *Cytospora* species on chinese chestnut in China. MycoKeys 62:1–25. https://doi.org/10.3897/mycokeys.62.47425

Jones WE (2008) Comparing virulence of *Cryphonectria parasitica* isolates recovered from portions of cultures or cankers established before versus those after hypovirus inoculations. Dissertation, West Virginia Universit

Kabbaj FZ, Lu S, Faouzi MA, Meddah B, Proksch P, Cherrah Y, Altenbach HJ, Aly A, Chadli A, Debbab A (2015) Bioactive metabolites from *Chaetomium aureum*: structure elucidation and inhibition of the Hsp90 machine chaperoning activity. Bioorg Med Chem 23:126–131. https://doi.org/10.1016/j.bmc.2014.11.021

Kolp M (2018) Fungal community succession and interactions in chestnut blight cankers on American chestnut trees in Michigan and Wisconsin. Dissertation. Michigan State University

Kolp M, Double ML, Fulbright DW, MacDonald WL, Jarosz AM (2020) Spatial and temporal dynamics of the fungal community of chestnut blight cankers on american chestnut (*Castanea dentata*) in Michigan and Wisconsin. Fungal Ecol 45:100925. https://doi.org/10.1016/j.funeco.2020.100925

Krstin L, Katanić Z, Ježić M, Poljak I, Nuskern L, Matković I, Idžojtić M, Ćurković-Perica M (2017) Biological control of chestnut blight in Croatia: An interaction between host sweet chestnut, its pathogen *Cryphonectria parasitica* and the biocontrol agent *Cryphonectria hypovirus* 1. Pest Manag Sci 73:582–589. https://doi.org/10.1002/ps.4335

Lione G, Danti R, Fernandez-Conradi P, Ferreira-Cardoso JV, Lefort F, Marques G, Meyes JB, Prospero S, Radócz L, Robin C, Turchetti T, Vettraino AM, Gonthier P (2019) The emerging pathogen of chestnut *Gnomoniopsis castaneae*: the challenge posed by a versatile fungus. Eur J Plant Pathol 153:671–685. https://doi.org/10.1007/s10658-018-1597-2

Lo Presti L, Lanver D, Schweizer G, Tanaka S, Liang L, Tollot M, Zuccaro A, Reissmann S, Kahmann R (2015) Fungal effectors and plant susceptibility. Annu Rev Plant Biol 66:513–545. https://doi.org/10.1146/annurev-arplant-043014-114623

Milgroom MG, Cortesi P (2004) Biological control of chestnut blight with hypovirulence: a critical analysis. Annu Rev Phytopathol 42:311–338. https://doi.org/10.1146/annurev.phyto.42.040803.140325

Nicoletti R, Beccaro GL, Sekara A, Cirillo C, Di Vaio C (2021) Endophytic Fungi and ecological fitness of Chestnuts. Plants 10:542. https://doi.org/10:542.10.3390/plants10030542

Oono R, Lefevre E, Simha A, Lutzoni F (2015) A comparison of the community diversity of foliar fungal endophytes between seedling and adult loblolly pines (*Pinus taeda*). Fungal Biol 119:917–928. https://doi.org/10.1016/j.funbio.2015.07.003

Pasche S, Calmin G, Auderset G, Crovadore J, Pelleteret P, Mauch-Mani B, Barja F, Paul B, Jermini M, Lefort F (2016) *Gnomoniopsis smithogilvyi* causes chestnut canker symptoms in *Castanea sativa* shoots in Switzerland. Fungal Genet Biol 87:9–21. https://doi.org/10.1016/j.fgb.2016.01.002

Potshangbam M, Devi SI, Sahoo D, Strobel GA (2017) Functional characterization of endophytic Fungal Community Associated with *Oryza sativa* L. and *Zea mays* L. Front Microbiol 8:325–330. https://doi.org/10.3389/fmicb.2017.00325

Prospero S, Lutz A, Tavadze B, Supatashvili A, Rigling D (2013) Discovery of a new gene pool and a high genetic diversity of the chestnut blight fungus *Cryphonectria parasitica* in caucasian Georgia. Infect Genet Evol 20:131–139. https://doi.org/10.1016/j.meegid.2013.08.009

R Core Team (2021) R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria

Robin C, Heiniger U (2001) Chestnut blight in Europe: Diversity of *Cryphonectria parasitica*, hypovirulence and biocontrol. For. Snow Landscape Res. 76:361–367

Rigling D, Prospero S (2018) *Cryphonectria parasitica*, the causal agent of chestnut blight: invasion history, population biology and disease control. Molr Plant Pathol 19:7–20. https://doi.org/10.1111/mpp.12542

Robbins NE (1997) Spread of white hypovirulent strains of *Cryphonectria parasitica* among American chestnut trees at the Lesesne State Forest. Dissertation. Virginia Polytechnic Institute and State University

Robin C, Lanz S, Soutrenon A, Rigling D (2010) Dominance of natural over released biological control agents of the chestnut blight fungus *Cryphonectria parasitica* in south-eastern France is associated with fitness-related traits. Biol Control 53:55–61. https://doi.org/10.1016/j.biocontrol.2009.10.013

Rodrigues P, Venâncio A, Lima N (2013) Incidence and diversity of the fungal genera *aspergillus* and *Penicillium* in Portuguese almonds and chestnuts. Eur J Plant Pathol 137:197–209. https://doi.org/10.1007/s10658-013-0233-4

RStudio team (2021) RStudio: Integrated Development Environment for R. RStudio Inc., Boston, MA

Schapovaloff ME, Alves LFA, Fanti AL, Alzogaray RA, Lastra CCL (2014) Susceptibility of adults of the cerambycid beetle *Hedypathes betulinus* to the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *purpureocillium lilacinum*. J Insect Sci 14127:93–100. https://doi.org/10.1093/jis/14.1.127

Solis MJL, Cruz TED, Schnittler M, Unterseher M (2016) The diverse community of leaf-inhabiting fungal endophytes from Philippine natural forests reflects phylogenetic patterns of their host plant species *Ficus benjamina*, *F. elastica* and *F. religiosa*. Mycoscience 57:96–106. https://doi.org/10.1016/j.myc.2015.10.002

Spatafora JW, Quandt CA, Kepler RM, Sung G-H, Shrestha B, Hywel-Jones NL, Luangsa-ard JJ (2015) New 1F1N Species Combinations in Ophiocordycipitaceae (Hypocreales). IMA Fungus 6:357–362. https://doi.org/10.5598/imafungus.2015.06.02.07

Suarez PC (1989) Fitopatologia del castaño (*Castanea sativa* Miller). Boletin de Sanidad Vegetal, Fuera de série, 16:1-130

Thom C (1930) The Penicillia. The Williams & Wilkins Company, Baltimore

Toksöz S, Oksal E, Saruhan I, Kepenekci I (2018) Pathogenicity of the entomopathogenic fungus, *purpureocillium lilacinum* TR1 against ambrosia beetles, *Xylosandrus germanus* (Blandford) and *Xyleborus dispar* (Fabricius) (Coleoptera: Curculionidade: Scolytinae). Mun Ent Zool 13:471–481

Wagenaar MM, Clardy J (2001) Dicerandrols, new antibiotics and cytotoxic dimmers produced by the fungus *phomopsis longicolla* isolated from an endangered mint. J Nat Prod 64:1006–1009. https://doi.org/10.1021/np010020u

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Shinsky J, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322

Yue Q, Miller CJ, White JF, Richardson MD (2000) Isolation and characterization of fungal inhibitors from *Epichloe festucae*. J Agric Food Chem 48:4687–4692. https://doi.org/10.1021/jf990685q

Zamora P, Martín AB, San Martín R, Martínez-Álvarez P, Diez JJ (2014) Control of chestnut blight by the use of hypovirulent strains of the fungus *Cryphonectria parasitica* in northwestern Spain. Biol Control 79:58–66. https://doi.org/10.1016/j.biocontrol.2014.08.005