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Rhizosphere mycobiome diversity in four declining Mediterranean tree species

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Introduction: Forests in the Mediterranean basin are currently in decline. Their resilience has been eroded as a result of climate change and anthropogenic impacts, making them vulnerable to increasingly frequent episodes of drought, fire and the spread of pests and diseases. The impact of these natural and anthropogenic events on soil biodiversity is of particular concern, as the soil fungal community plays a key role in ecosystem homeostasis.

Objectives and methods: In order to analyse the relationship between soil health status and fungal diversity, soil samples were collected from declining Mediterranean forests of *Castanea sativa* (chestnut), *Quercus ilex* (holm oak), *Quercus suber* (cork oak) and *Quercus pyrenaica* (Pyrenean oak). A metabarcoding study was carried out by sequencing the ITS genomic region.

Results: A total of 674 fungal genera were found. It has not been possible to explain the differences in health status from the fungal genera found exclusively on declining forest soils, as none of them have been described as pathogenic. Healthy chestnut soils were characterized by a high alpha diversity and a higher abundance of the genus *Metarhizium*. No differentially abundant genera were found in any of the other forest species tested. Declining chestnut soils harbored more abundance of ectomycorrhizae and soil saprotrophs than healthy samples. Ectomycorrhizae were the dominant lifestyle in all oak species regardless of health status, whereas arbuscular mycorrhizae were preferentially found in declining cork oak soils.

Discussion: This work highlights the resilience of fungal communities of soil against decline and highlights the need to further investigate its relationship with the forest's ability to cope with the challenges of climate change.

KEYWORDS

global change, forest declines, metabarcoding, biodiversity, forest pathology, *Quercus*, *Castanea*

1. Introduction

Mediterranean forests are characteristic ecosystems of the Mediterranean Basin, but also present in California, South Africa and Chile. These forests are a hot spot of genetic diversity in the European continent, as they harbor 290 tree species, compared to 135 reported in the rest of Europe (Gauquelin et al., 2018). The organisms that make up the Mediterranean forests are usually adapted to very specific climatic factors, such as the existence of a marked seasonality

characterized by long periods of summer drought, and great variability in the total annual rainfall. In addition, Mediterranean forests have been subjected for millennia to anthropogenic impacts, mainly fragmentation due to land use change toward agriculture and grazing, overexploitation to obtain goods and services and, more recently, abandonment of their use (Fady et al., 2022). These characteristics have confirmed that Mediterranean forests are as vulnerable to management policies as they are to climate change (Morán-Ordóñez et al., 2020).

Some of the main tree species found in Mediterranean forests are chestnut, cork oaks, holm oaks and Pyrenean oaks. Chestnut (*Castanea sativa* Mill.) covers more than 2.5 million hectares in the Mediterranean Basin, being the only species of the genus native to this region. Chestnut has been widely cultivated as a monoculture for centuries and as a source of timber and fruit, although in recent decades it has been progressively felled (Garfì et al., 2022). Cork oak (*Quercus suber* L.) spreads over between 1.7 and 2.7 million ha of the Mediterranean Basin and has been cultivated for 3,000 years for the production of bark, representing the second most important marketable non-timber forest product (Gauquelin et al., 2018). Holm oak (*Quercus ilex* L.) forests comprise 4% of the forests of the Mediterranean Basin, being fully adapted to a wide range of soil and climatic conditions. In spite of the continuous anthropic exploitation of holm oaks during several centuries, the reduction in the use of their wood as fuel in the last 50 years is turning holm oak forests into high forests (Camponi et al., 2023). Pyrenean oak (*Quercus pyrenaica* Willd.) is found only in France, Spain, Portugal and Morocco. Pyrenean oak forests have historically been exploited by man for timber, and still are to this day, and constitute the main ecosystem of the Mediterranean Basin in diversity of hoverfly species (Perez-Luque et al., 2020; Martín-Pinto et al., 2023).

At present, the Mediterranean forest is in a situation of regression and dieback in several areas of the Mediterranean Basin, due to increased periods of drought, overexploitation, fire, soil degradation and the spread of pests and diseases (Peñuelas and Sardans, 2021). The decline of Mediterranean forests is a complex phenomenon due to multiple and very different causes that can occur slowly and over several years, ending with tree death (Gentilesca et al., 2017). In the case of chestnuts, drought (Waldboth and Oberhuber, 2009), or pathogens such as *Cryphonectria parasitica* (Elliott and Swank, 2008; Waldboth and Oberhuber, 2009), or *Phytophthora* sp. (Vettraino et al., 2005; Akıllı et al., 2012) have been reported as causes related to decline. In cork oak, causes include drought (Zribi et al., 2016; Camilo-Alves et al., 2017; El Ahmadi et al., 2022) and fungi such as *Phytophthora* sp. (Smahi et al., 2017). Or in the case of holm oak, drought events (Limousin et al., 2009) or pathogens such as *Phytophthora cinnamomi* (Frisullo et al., 2018) have been associated with decline.

Soil fungi play fundamental roles in the functioning of ecosystems, as decomposers of organic matter and as beneficial and detrimental organisms in interaction with plants (Wardle and Lindahl, 2014; Fernandes et al., 2022). In the study of soil mycobiome diversity, the DNA metabarcoding methodology represents one of the most widely used tools in the last decade (Wardle and Lindahl, 2014; Tedersoo et al., 2021). Precisely, metabarcoding has identified how disturbance events of natural and anthropogenic origin have a significant effect on the soil mycobiome of forests (Bowd et al., 2022; Martín-Pinto et al., 2023). This methodology of mycobiota diversity analysis has already been used in Mediterranean forest, being described as an effective way for the real knowledge of microbial diversity and functionality (Adamo et al., 2021a,b), also in situations of disturbance, such as fire (García-Carmona et al., 2021) or climate change (Diez-Hermano et al., 2022).

In chestnut, the study of soil fungal diversity by metabarcoding has been confirmed as a more comprehensive strategy than the study of fruiting bodies (Baptista et al., 2015). In the presence of the pathogenic oomycete *Phytophthora cambivora*, it has been reported by metabarcoding how soil mycobiota show resilience to the pathogen, and may play a key role in the forest's resistance to the disease (Venice et al., 2021). The use of metabarcoding in the study of rhizospheric mycobiota of cork oaks has revealed the influence of abiotic factors, such as climate, on its composition (Costa et al., 2022). In addition, the diversity of soil mycobiota is closely related to the decline of cork oaks (Maghnia et al., 2019). In this sense, the fungal diversity most studied by metabarcoding in relation to decline is that of the holm oaks of Spanish "dehesas." In this way, it has been possible to identify how pathogens such as *Phytophthora cinnamomi* in combination with others (such as *Fusarium* spp., *Alternaria* spp. or *Pythium spiculum*) and not in isolation are involved in the development of decline, as well as how fungi such as *Trichoderma* spp. or ectomycorrhizae improve the response of trees affected by these pathogens (Ruiz-Gómez et al., 2019a,b). With respect to Pyrenean oak, to our knowledge, the study of its rhizospheric/soil mycobiota by metabarcoding has been little addressed so far. For example, it has been possible to relate how different management strategies have an impact in soil fungal diversity (Martín-Pinto et al., 2023).

Therefore, the aim of this work is to relate soil mycobiota with the health status of Mediterranean forests. To this end, chestnut, cork oak, holm oak and Pyrenean oak have been selected as key forest species, obtaining rhizospheric soil samples from healthy and declining trees in forests with known presence of pests and pathogens. Soil mycobiota was analyzed by metabarcoding using the ITS region of ribosomal DNA.

2. Materials and methods

2.1. Sampling sites and procedure

Soil samples were extracted from the rhizosphere of healthy and declining tree of *Castanea sativa* (chestnut), *Quercus suber* (cork oak), *Quercus ilex* (holm oak) and *Quercus pyrenaica* (pyrenean oak) from Salamanca (Castile and Leon, Spain) forests (Table 1). Each site sampled corresponded to a single tree species. Soil collection was conducted for all four sites in June and July 2020.

For all sampling sites, declining patches were defined as areas with high percentage of declining trees (presence of canker wounds or stem bleeds, >70% of trees with severe dieback and foliage wilting). Health condition was assessed visually from the ground following guidelines from the ICP Forests Manual Part IV "Visual Assessment of Crown Condition and Damaging Agents" (Eichhorn et al., 2016). Stands of healthy trees were primarily composed of asymptomatic trees free of dieback or crown transparency. Data on tree assessment as well as a map of sampling locations can be found in Supplementary Figures S1, S2.

Three circular plots of healthy trees and three with high degree of decline were selected in each site. Distance between sampled trees

TABLE 1 Description of sampling sites.

Location	Size and density	Characteristics	Soil features	Sampled species	Main criteria for decline
La Alamedilla, Spain	60 ha, 49 trees/ha	Elevation: 756 m Rainfall: 600–854 mm	Medium soil with sandy loam texture and high organic matter content	*Quercus ilex*	Dead trees and exudates
Linares de Riofrío, Spain	10 ha, 1,250 trees/ha	Elevation: 956 m Rainfall: 700–900 mm	Gravels in coarse textured areas and very high organic matter content	*Castanea sativa*	Dead trees and cankers
Cubo de Don Sancho, Spain	6,000 ha, 24 trees/ha	Elevation: 736 m Rainfall: 500–700 mm	Gravels in coarse textured areas and medium organic matter content	*Quercus pyrenaica*	Dead trees, dieback and exit holes
Valdelosa, Spain	6,000 ha, 78 trees/ha	Elevation: 842 m Rainfall: 500–700 mm	Gravel and phreatic zones with sandy loam texture and low organic matter content	*Quercus suber*	Dieback and crown transparency

ranged between 10 and 50 m. Soil under the canopy of five live trees was sampled in and pooled per plot. Surface debris was removed and four cores (100 cm^3 each at opposite N, S, W, E cardinal points) of topsoil from underneath the litter layer were collected, around one meter from each tree trunk. Coarse roots and stones were removed. All soil cores from each plot were pooled, resulting in a composite soil sample per plot and totalling 24 samples (4 sampling sites × 3 plots × 2 health conditions). Samples were stored at −20°C prior to processing.

Throughout this paper soil sampled from stands of healthy trees will be referred to as "healthy trees soil" whereas soil from stands of declining trees will be termed "declining trees soil."

2.2. Sample processing and sequencing

Physicochemical analyses of soil samples were performed in triplicate, at the Analysis and Instrumentation Service of IRNASA-CSIC by standard methods. Analyzed soil properties can be found in Supplementary Table S1.

Samples were sent for molecular analysis to Base Clear B.V (Leiden, Netherlands). DNA extraction was performed using the DNeasy PowerLyzer PowerSoil kit (Qiagen). Region 2 of fungal Internal Transcribed Spacer (ITS2) gene was amplified using the following primers: ITS7-F: 5′-GTG ART CAT CGA RTC TTT G-3′, ITS4-R: 5'-TCC TCC GCT TAT TGA TAT GC-3′ (Fujita et al., 2001; Ihrmark et al., 2012). Paired-end sequence reads (2×300 bp) were generated using the Illumina MiSeq V2 system under accreditation according to the scope of BaseClear B.V (L457; NEN-EN-ISO/IEC 17025). FASTQ read sequence files were generated using bcl2fastq2 version 2.18. Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing PhiX control signal were removed using an in-house filtering protocol. In addition, reads containing (partial) adapters were clipped (up to a minimum read length of 50 bp). The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.11.5. ITS2 sequences were processed following the DADA2 pipeline to generate amplicon sequence variants (ASV) (Callahan et al., 2016). Parameters' values were as follows: filtering and trimming (maxN = 0, maxEE = 2, truncQ = 2, minLen = 50, rm.phix = TRUE, compress = TRUE), learning error rates (nbases = 1e+08, nreads = NULL, errorEstimationFunction = loessErrfun, MAX_CONSIST = 10, OMEGA_C = 0), merging paired reads (errorEstimationFunction = loessErrfun, selfConsist = FALSE, pool = FALSE) and removing chimeras (method = "consensus"). Taxonomy assignment and abundance estimation were performed comparing ASVs against UNITE database version 9.0 (Abarenkov et al., 2022). Rarefaction curves were used to evaluate the relationship between sequencing depth and the number of ASVs.

2.3. Statistical analysis

Prior to the analysis, taking into consideration that only ITS2 marker was used, ASVs' raw reads were aggregated at genus level to have better confidence, and to avoid misidentification of closely-related species (Yang et al., 2018).

Differences between mycobiome communities were evaluated in terms of alpha and beta diversity. Alpha diversity was assessed using Hill diversity indexes according to the equation

$$D = \left(\sum_{i=1}^{S} p_i \left(r_i \right)^l \right)^{\frac{1}{l}}$$

where D is diversity, S is the number of taxa, p_i is the proportion of all individuals that belong to taxa i, r_i is the rarity of taxa i, defined as $1/p_i$, and l is the exponent that determines the rarity scale and corresponds to richness ($l = 1$) or equivalence-corrected versions of Shannon ($l = 0$) and Simpson indexes ($l = -1$) (Roswell et al., 2021). Differences between health conditions per tree species at $l = [-1, 0, 1]$ were contrasted using Wilcoxon's rank test.

Beta diversity was evaluated in terms of differential abundance by taking into account that high-throughput sequencing counts should be considered compositional data (Gloor et al., 2017). Analyses followed the ZicoSeq procedure, which has been shown to be overall more robust and powerful than other existing methods in recent benchmarks (Yang and Chen, 2022). Compositional effects were addressed by adopting a reference-based approach (selecting close-to-invariant taxa as baseline abundances) and association testing was conducted by linear model-based Smith permutation testing [LDM and DACOMP methods in Brill et al. (2022) and Hu and Satten (2020), respectively]. Reference taxa were adjusted for health status (factor with two levels: healthy, declining) as a covariate. Taxa were filtered if their prevalence was less than 20% and their mean relative abundance was less than 0.2%. Percentage of top outliers replaced by

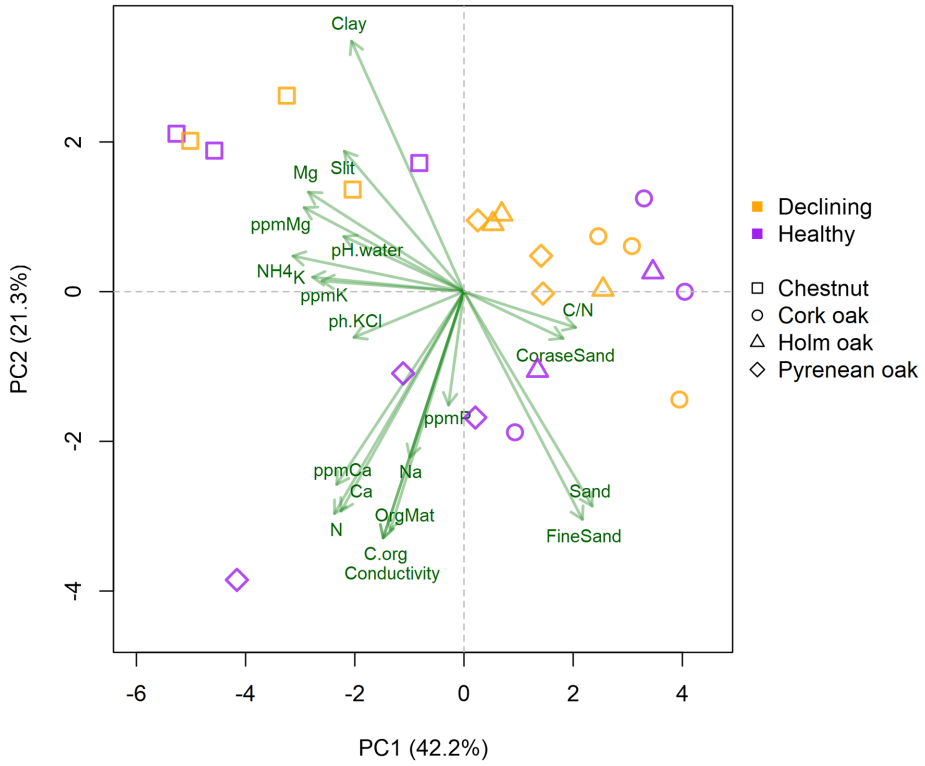

FIGURE 1
Biplot of soil properties, tree species and health status. Green arrows represent soil properties and colored symbols represent soil samples. Axis correspond to the first and second principal components, accounting for 63.5% of total variance. Variables were scaled prior to analysis.

winsorization was 10%. Abundances were square root transformed. Multiple test correction of *p*-values was based on 500 permutation tests.

All fungal genera were included in the functional analyses. Each genus was assigned a functional guild according to the "primary lifestyle" column obtained from FungalTraits database V1.2 (Põlme et al., 2020). Raw read numbers of each guild were summed per tree species and health condition and expressed as $\log_2$(guild abundance/total abundance).

All analyses were performed in R environment 4.1.3 (R Core Team, 2022). Analysis of sequencing data and ASV identification were performed using the packages *Biostrings* (Pagès et al., 2022), *dada2* (Callahan et al., 2016) and *ShortRead* (Morgan et al., 2009). Hill diversity analysis was carried out using the package *MeanRarity* (Roswell and Dushoff, 2022). For compositional analysis the package *GUniFrac* (Chen et al., 2022) was used. Taxonomic information was handled and plotted with the package *metacoder* (Foster et al., 2017).

3. Results

3.1. Soil description

Physico-chemical properties of soils were analyzed and summarized using Principal Component Analysis (Figure 1). Chestnut's soils were characterized by clay and slit texture, higher content of assimilable and free Mg and K and a slight tendency to acidity with high concentration of ionized NH4+. Oak's soils were characterized by sandy textures and a higher C/N ratio, except for soils from declining Pyrenean oaks, which were enriched in Ca, N, P and organic matter. No further associations between health status and soil properties were observed.

3.2. Fungal community description

Sequencing yielded 32,042 (29590–33,305) reads on average (median and P25-75) following quality control (Supplementary Table S2). All samples showed plateauing rarefaction curves (Supplementary Figure S1). The analysis of the fungal communities reported 674 different genera distributed in 15 phyla (Supplementary Table S3). The majority of the phyla were Ascomycota (~66%) and Basidiomycota (~26%) (Figure 2).

To analyse the relationship between the presence/absence of certain fungal genera and forest health status, a comparison was made without considering the plant species. Only those genera that were present in at least 80% of the samples were taken into account. Twenty-two fungal genera (68.8%) were found common to healthy and declining trees soils, while six genera (18.8%) were only present in healthy trees soils and four genera (12.5%) were exclusive to declining forest soils. The genera found in healthy forest soils were *Coleophoma, Geomyces, Podila, Sagenomella, Scytalidium,* and *Varicellaria*. On the other hand, the fungal genera found in declining trees soils, which did not appear in samples of healthy trees, were *Humicola, Meliniomyces, Pleurotus* and *Polyphilus* (Figure 3).

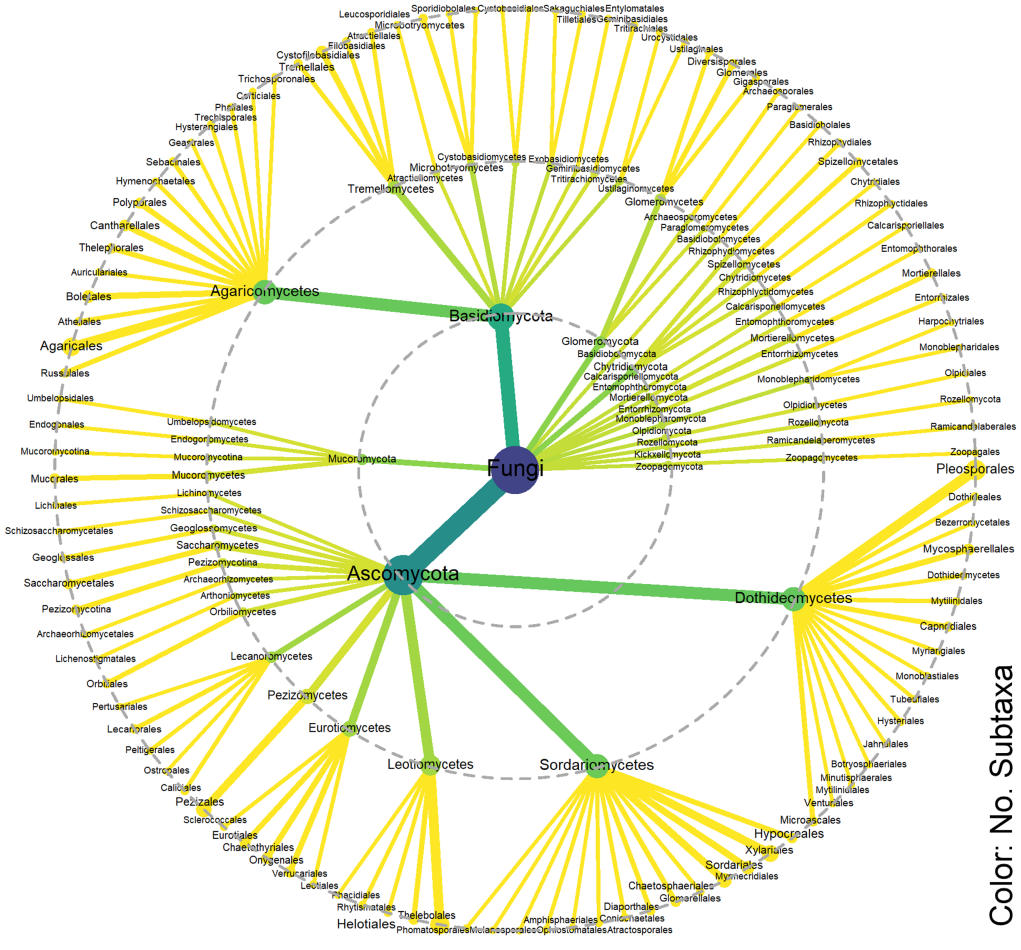

FIGURE 2
Taxonomic heat tree. Node size represents the number of OTUs and color scale represents the number of subtaxa. Dashed grey circles indicate the following taxonomic levels: phylum (inner), class (middle), order (outer). NA: not assigned.

3.3. Diversity analysis

Diversity analysis was performed by comparing the effect of decline in the different tree species. In chestnut there was significantly higher alpha diversity in soil from healthy trees than in soil from declining trees. On the other hand, both cork oak and holm oak reported higher alpha diversity in soil from declining trees than in healthy trees; specifically, the diversity was significantly higher for dominant fungal genera in cork oak (Hill = −1) and for rare genera in holm oak (Hill = 1). In the case of Pyrenean oak, no significant differences were found between fungal genus diversity in soil from healthy and declining trees (Figure 4).

Beta diversity was evaluated in terms of differential abundance (DA) of fungal compositions using square root-transformed values of raw reads. In cork oak, holm oak and Pyrenean oak, no fungal genus was found significantly more abundant in soils of declining trees, nor in healthy trees (Figure 5). Only the genus *Metarhizium* was found to be significantly more abundant in soils of healthy chestnuts (p-val = 0.1 and R2 = 0.2). However, no fungal genus was reported to be significantly more abundant in declining chestnut soils.

3.4. Functional profiles

Primary lifestyle was assigned to each of the fungal genera reported in healthy and declining forest soils per tree species. Most prominent functional niches identified were ectomycorrhizae (EcM) and soil saprophytes, followed by litter/wood saprophytes and plant pathogens, regardless of tree species (Figure 6). Root endophytes were also particularly abundant in soil from holm oak and Pyrenean oak. When taking health status into account, the biggest differences were found in chestnuts and cork oaks, with holm oaks and Pyrenan oaks having similar functional profiles between healthy and declining trees' soil. Fungal genera whose primary function is to form part of the lichen symbiosis were found to be more abundant in healthy chestnut and cork oak soils than in declining soils. Sooty mold fungi in chestnut soils followed the same trend. In cork oaks the largest differences corresponded to arbuscular mycorrhizae (AM), more present in declining than in healthy trees' soil. Some lifestyles were absent from the majority of samples, such as moss symbiont, protistan parasites, epiphytes and algal parasites.

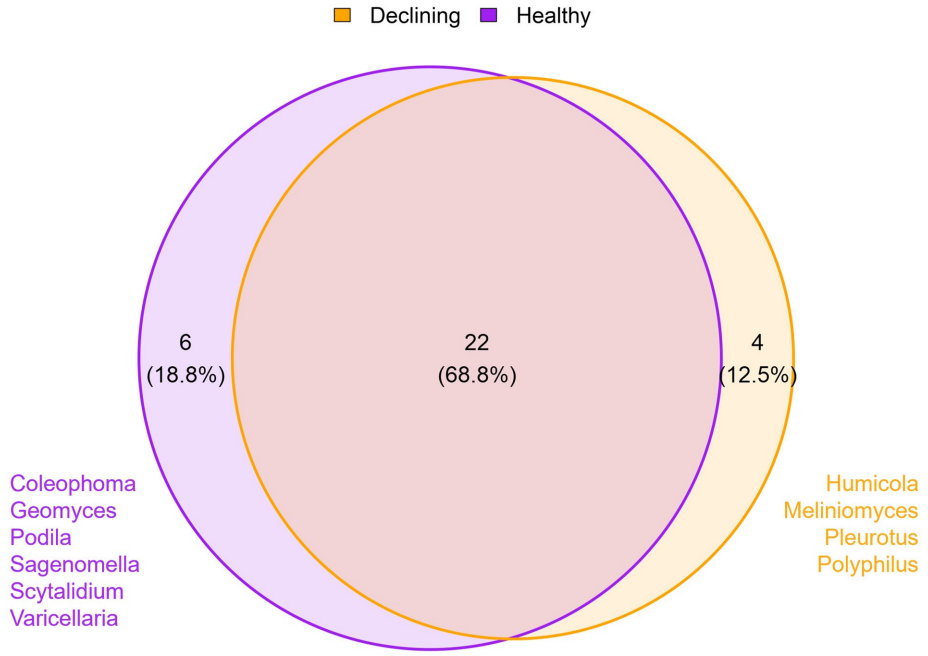

FIGURE 3
Overall effect of health status on mycobiota. Venn's diagram showing the number of common and exclusive genera between samples from healthy ($n = 12$) and declining ($n = 12$) trees. Only genera present in 80% of samples per condition are included, regardless of tree species.

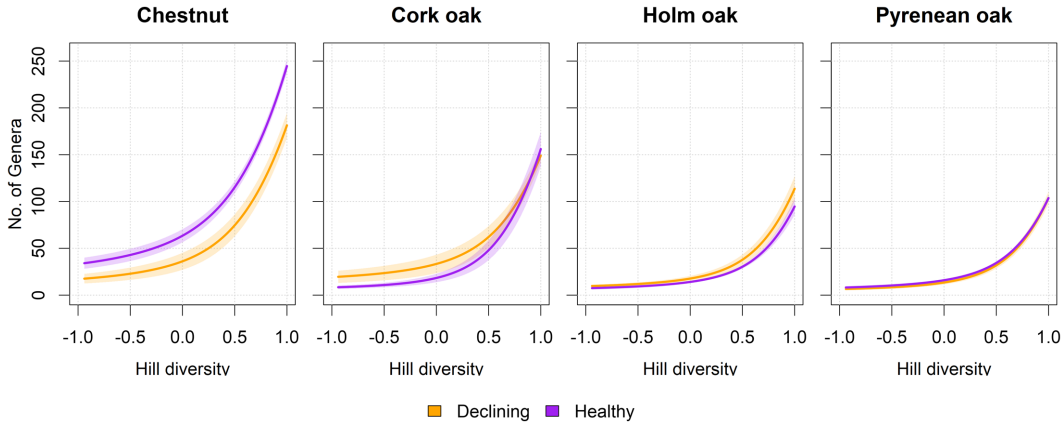

FIGURE 4
Diversity profiles per tree species and health status. Curves represent the average diversity in samples from healthy and declined trees in terms of an imaginary assemblage with that same diversity, but in which all species are equally abundant (Jost, 2006; Roswell et al., 2021). The horizontal axis represents the exponent l of Hill diversity, which can be interpreted as equivalence-corrected versions for richness ($l = 1$), Shannon ($l = 0$) and Simpson ($l = -1$) diversity estimators. Shadowed intervals correspond to standard error. Significant differences were found at $l = [1, 0, -1]$ between declining and healthy chestnut soils (Wilcoxon test, $n = 3$ per tree species and health condition, p-val < 0.05). Raw curves prior to averaging can be found in Supplementary Figure S2.

4. Discussion

Soil mycobiota play a key role in forest functioning and health (Fernandes et al., 2022). Therefore, knowing the fungal diversity of forest soils under different situations and health status is fundamental to understand the role that each component of this diversity plays in the sustainability of the ecosystem, being metabarcoding a proven tool for this purpose (Bowd et al., 2022). In our study, this methodology has allowed us to identify 674 different fungal genera as inhabitants of the rhizosphere of chestnuts, cork oaks, holm oaks and Pyrenean oaks.

Some fungal genera were only present in soils of declining trees. However, none of these genera has been previously described as a forest pathogen, which might be indicative of the soil conditions playing an important role as a predisposing or inciting factor of decline *per se*. *Humicola* is a genus of endophytic fungi of great biotechnological interest in recent years, mainly related to halophytic plants and production of chemical compounds for industrial use (Hosseyni-Moghaddam et al., 2020). *Meliniomyces* is a genus of endophytic fungi and ericoid mycorrhiza (Ohtaka and Narisawa, 2008; Vohnik et al., 2013). *Pleorotus* is a genus of widely known edible

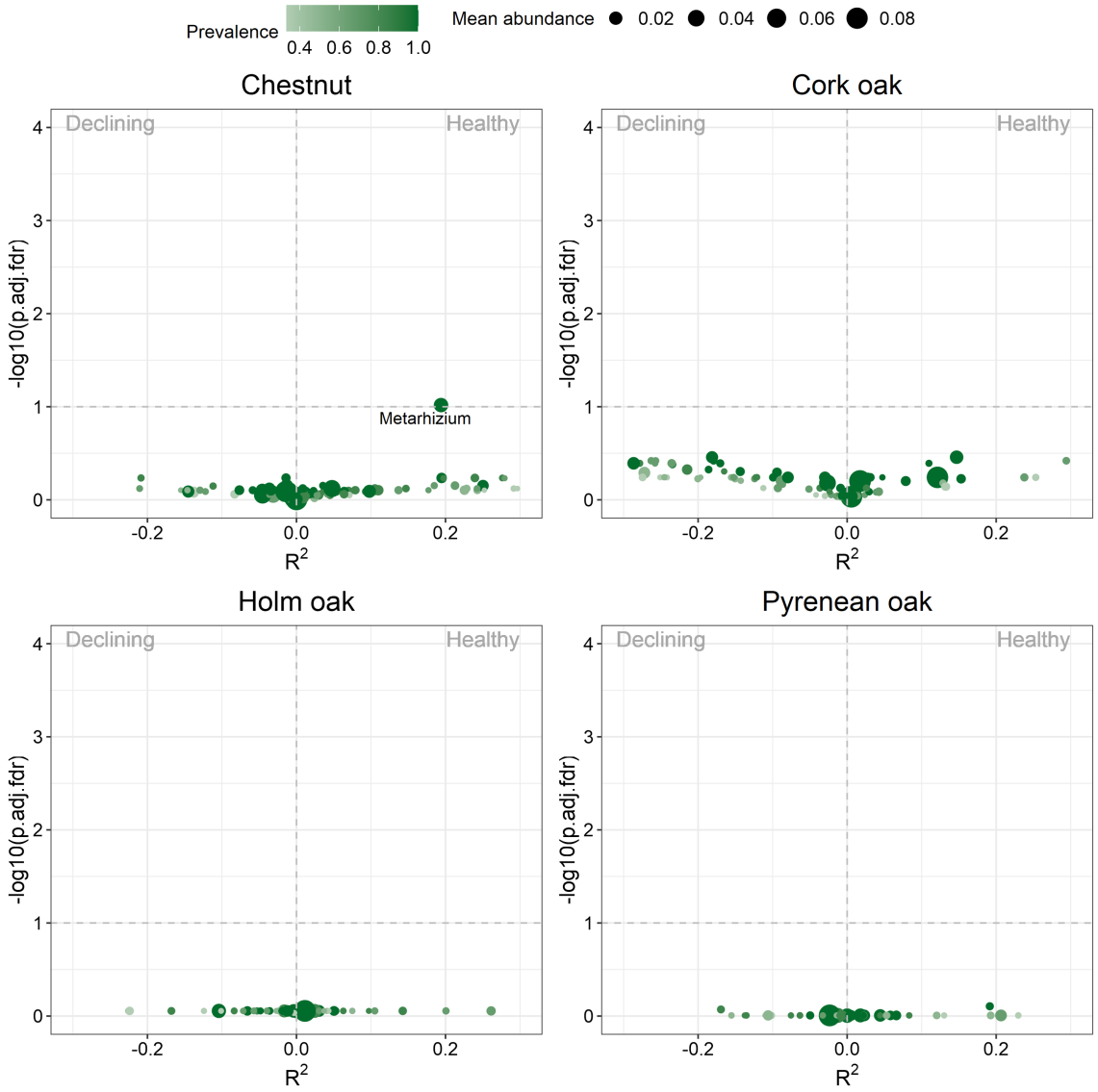

FIGURE 5
Volcano plot of differentially abundant genera detected by ZicoSeq analysis. Each genus is depicted by a dot. Dot size indicates the mean abundance across all samples and color indicates the proportion of samples in which the genus is present. Vertical axis represents value of p adjusted by false discovery rate (FDR) in logarithmic scale (1 = 0.1, 1.30 = 0.05, 2 = 0.01 and so on). Horizontal axis represents the strength of association between abundance and health condition, with the sign indicating the association direction (negative for declining, positive for healthy). Genera that surpass the horizontal dashed line are differentially abundant (p-val < 0.1).

saprophytic fungus (Suwannarach et al., 2020; Doroški et al., 2022) which also includes biological control agents against nematodes (Singh et al., 2019), and its presence is most likely tied to clearings in declining areas. *Polyphilus* is a recently created fungal genus, which includes nematophagous fungi of eggs and cysts of plant-parasitic nematodes (Ashrafi et al., 2018). However, other important microbial communities with putative pathogenic species and capable of exerting direct and indirect effects on these forest species, such as bacteria, oomycetes or nematodes, have not been addressed here. Future work would be interesting in this regard, and could provide important information on their relationship with the health status of the trees as well as their impact on population dynamics of non-pathogenic species.

Similarly, we also found fungal genera exclusive to healthy forest soils, which could be related to control activity of different pathogens. *Varicellaria* is a fungal genus whose life form is algal symbiosis to form lichens (Schmitt et al., 2012) and could be related to the presence of more humidity due to better vegetative condition of the trees. A few species of *Podila* have been described as mycophilic fungi or mycoparasites (Telagathoti et al., 2022), but the absence of specific studies on their potential biocontrol effect prevents associating their presence with the absence of decline. Some genera found can act as animals' pathogens, such as the genus *Geomyces* (Warnecke et al., 2012). The genus *Sagenomella* is widely known to cause systemic illness in animals (Gené et al., 2003), whereas the genus *Scytalidium* includes the causal agent of scytalidiosis and onychomycosis, human skin diseases (Elewski, 1996; Machouart et al., 2013). Interestingly, the genus *Coleophoma*, also absent from declining samples, has been widely described as a forest pathogen (Pehl and Wulf, 2003;

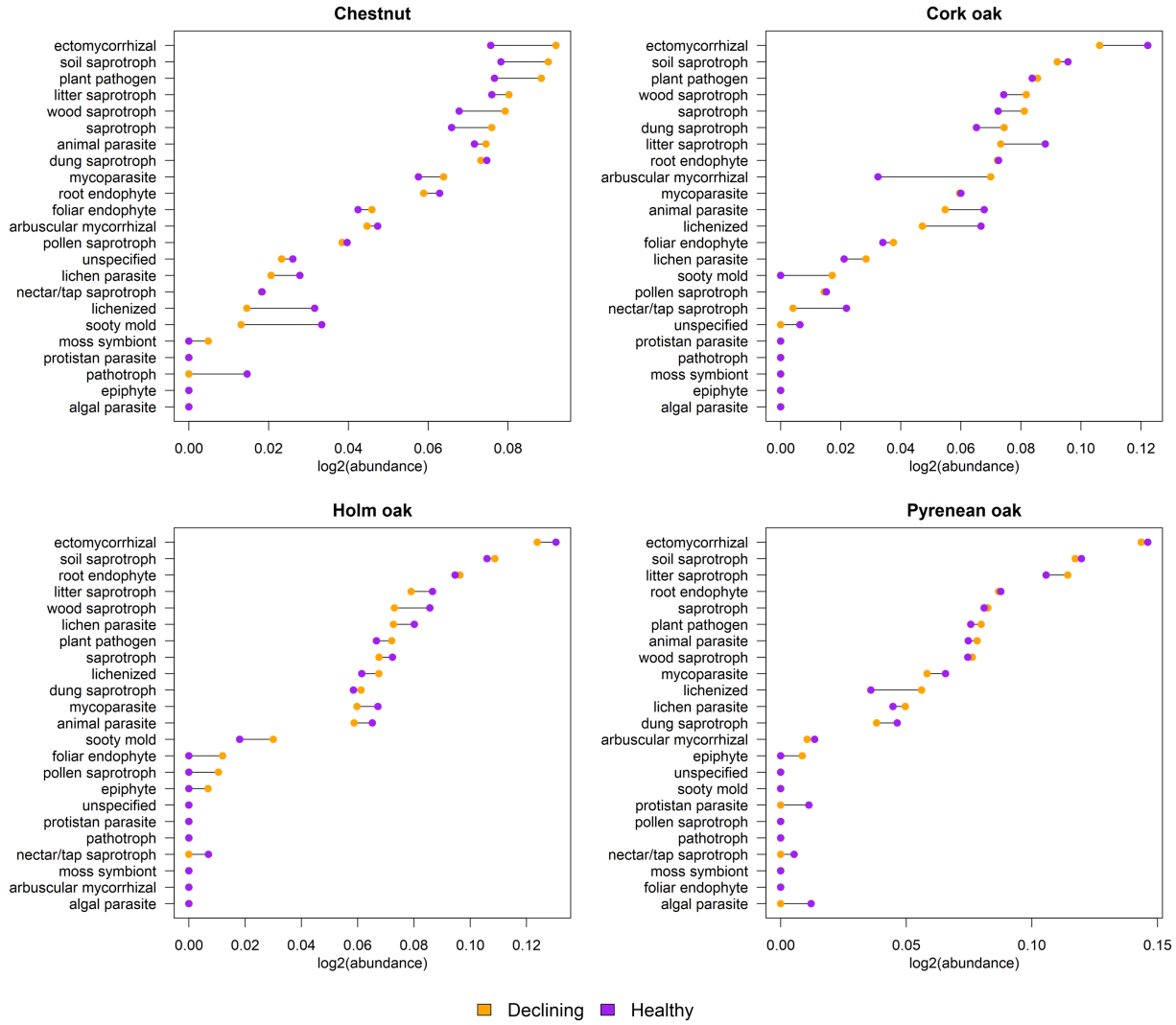

FIGURE 6
Comparison of functional guilds between declined and healthy samples. Horizontal axis represents sum of abundances expressed as $\log_2$ (guild abundance/total abundance). Connecting lines between two dots correspond to the difference between declined and healthy samples.

Bianchinotti and Rajchenberg, 2004). Grazing activities that take place regularly in the sampling sites could offer an explanation to the presence of fungi pathogenic to animals, although their association with healthy trees soils would point to a preference of cattle for such areas that has not been explored.

Regarding biodiversity, a higher alpha diversity was observed in soils of healthy chestnuts than in declining trees. In this sense, other authors have previously described that the rhizosphere of chestnuts has a high diversity, even much higher than that of other forest ecosystems (Kelly et al., 2021). Moreover, this fungal diversity plays a key role in the resistance of trees against pathogens such as the oomycete *Phytophthora cambivora* (Venice et al., 2021). On the other hand, in both cork oak and holm oak, we found a higher fungal diversity in the rhizosphere of declining trees than in healthy ones. Therefore, a higher fungal diversity in these trees may not be related to the biological control of the decline, as has been described by other authors (Gómez-Aparicio et al., 2022). Finally, no differences in alpha diversity were reported between soils of healthy and declining Pyrenean oaks, a novel aspect that requires further studies. Differential abundance analysis indicated that the genus *Metarhizium* is more present in healthy chestnut soils than in declining soils. *Metarhizium* has been widely described as a biological control agent against plant pests and pathogens (Stone and Bidochka, 2020), which might be indicative of this genus playing a significant role in the absence of declining in the selected healthy chestnut stands. Influence of environmental drivers on biodiversity such as slope or elevation has not been evaluated and would be an important confusion factor to be considered in future works.

With respect to the assignment of primary lifestyles, most common functional niches found were EcM and soil saprophytes, very widespread and important functions in fungi that make up the mycobiota of all forest soils (Li et al., 2022). EcM fungi were dominated by some ubiquitous genera such as *Cenococcum, Cortinarius, Inocybe, Laccaria, Russula* and *Tomenteria*, with *Russula* being particularly more abundant in holm oak and Pyrenean oak soils regardless of health status (see complete list in Figure S5). In general, all oak species shared a

predominance of EcM over other functional guilds. Inverse relationship between abundance of EcM and abundance of other guilds has been found before in oaks' soils (Ruiz Gómez et al., 2019b). In contrast, chestnut showed evenness of EcM, saprotrophs and pathogens. We hypothesize that these differences in balance of functional guilds between oaks and chestnuts could be related to oaks' soils being generally poorer and dryer and thus more prone to prevalence of EcM than chestnuts' soils, where water is more readily available to the plant. The aforementioned guilds were also found to be more abundant in declining chestnut soils. Higher presence of opportunistic pathogens is to be expected in the rhizosphere of weakened trees, whereas saprobes might benefit from the excess of dead matter and leaf litter. Declining chestnuts could also have increased generation of roots as an attempt to compensate for compromised water transport, which in turn would allow for more colonization of EcM, however, this phenomenon was not observed in the oak species. Regarding other lifestyles, a significant proportion of AM belonging to 15 genera was found in chestnut and declining cork oak soils, whereas holm oak, Pyrenean oak and healthy cork oak soils showed a remarkable absence of AM (Supplementary Figure S5). In particular, presence of some genera such as *Archaeospora, Entrophospora* and *Dominikia* was tied to health status in cork oak soils, favoring declining samples. The persistence of AM has been reported in dying succulent trees in correlation with $NO3^-$ levels in soil (Vivas et al., 2018), although we found no differences in nitrogen compounds between healthy and declining cork oak soils in our samples.

This work describes the fungal soil mycobiota of four Mediterranean forest species and explores differences between healthy and declining stands. Healthy chestnut soils were characterized by higher alpha diversity and higher abundance of the genus *Metarhizium*, but such relationships were not found in the rest of the forest species included in the study. Finally, with respect to the functional profile, declining chestnut soils harbored more abundance of EcM, saprobic and pathogenic guilds than healthy samples. EcM were the dominant lifestyle in all oak species regardless of health status, whereas AM were preferentially found in healthy cork oak soils. Further experimental validations would be necessary to assess whether functional shifts in soil mycobiota are causally related to trees' health status.

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 at: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA953856 https://github.com/serbiodh/2023_FrontiersForests_Soil, GitHub.

Author contributions

IB-A collected the data. SD-H analyzed the data. JP, SD-H, and JD wrote the manuscript. IB-A, JN-S, PM-P, AP, and JD designed the sampling scheme. All authors contributed to the article 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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2023.1215701/full#supplementary-material

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