forests

MDPI

Article

Fungal Perspective of Pine and Oak Colonization in Mediterranean Degraded Ecosystems

Irene Adamo [1,2], Svetlana Dashevskaya [2] and Josu G. Alday [1,2,*]

[1] Joint Research Unit CTFC-AGROTECNIO-CERCA, Av. Alcalde Rovira Roure 191, E25198 Lleida, Spain; irene.adamo@udl.cat
[2] Department Crop and Forest Sciences, University of Lleida, Av. Alcalde Rovira Roure 191, E25198 Lleida, Spain; svetlana.dashevskaya@udl.cat
* Correspondence: josu.alday@udl.cat or josucham@gmail.com

Abstract: Forest restoration has become one of the most important challenges for restoration ecology in the recent years. In this regard, soil fungi are fundamental drivers of forest ecosystem processes, with significant implications for plant growth and survival. However, the post-disturbance recovery of belowground communities has been rarely assessed, especially in highly degraded systems such as mines. Our aim was to compare forests and mined systems for biomass and structure of fungal communities in soil during early stages of tree establishment after disturbance. We performed ergosterol analysis and PacBio and Illumina sequencing of internal transcribed spacer 2 amplicons across soil layers in *P. sylvestris*, *Q. robur* and *Q. ilex* (holm oak) forests and naturally revegetated mined sites. In pine forests, total fungal biomass was significantly higher in litter and humus compared to mineral layers, with dominance of the mycorrhizal genera *Tomentella*, *Inocybe* and *Tricholoma*. Conversely, in oak forests the most abundant mycorrhizal genera were *Tomentella*, *Cortinarius* and *Sebacina*, but the biomass of saprotrophic fungi was greater in the litter layer compared to mycorrhizal fungi, with the genus *Preussia* being the most abundant. In the revegetated mined sites, ectomycorrhizal fungi dominated in the humus and mineral layers, with the mycorrhizal genus *Oidiodendron* being dominant. In contrast, in holm oak forests saprotrophic fungi dominated both soil humus and mineral layers, with the genera of *Alternaria*, *Bovista* and *Mycena* dominating the soil humus forest layer, while the genus *Cadophora* dominated the mineral layer. The habitat-specific differences in soil fungal community composition and putative functions suggest that an understanding of soil–plant–microbial interactions for different tree species and use of specific soil/litter inoculum upon planting/seeding might help to increase the effectiveness of tree restoration strategies in Mediterranean degraded sites.

Keywords: Forest expansion; soil fungi; *Pinus* and *Quercus* forest; plant soil feedback; mycorrhizal; saprotrophs

Citation: Adamo, I.; Dashevskaya, S.; Alday, J.G. Fungal Perspective of Pine and Oak Colonization in Mediterranean Degraded Ecosystems. *Forests* **2022**, *13*, 88. https://doi.org/10.3390/f13010088

Academic Editor: Ari M. Hietala

Received: 28 October 2021
Accepted: 5 January 2022
Published: 8 January 2022

1. Introduction

Forests cover 31% of the global land area and are home to most of Earth's terrestrial biodiversity [1], being fundamental as water-catchments and in the storage of carbon stocks to mitigate the effects of climate change [2]. Unfortunately, the increase in frequency and intensity of man-made disturbances has favoured deforestation and forest degradation at alarming rates in some parts of the world [3]. Consequently, forest restoration has become one of the most important challenges facing restoration ecology in recent years [4]. The re-establishment of tree species in degraded areas is usually a difficult task, mainly due to the environmental hardness and biotic and abiotic soil limitations of disturbed ecosystems [5]. The re-establishment of trees has been focused on the identification of suitable microsites and mechanisms that facilitate the tree seedling survival and growth [4], to regenerate the structure, productivity, and plant species community composition [6]. However, notably absent from the forest restoration framework is the consideration of how the interactions

and feedbacks between above- and below-ground biological communities and abiotic conditions underpin critical ecological functions and biogeochemical processes [7,8].

Below-ground microbial communities are important drivers of ecosystem functions such as decomposition, nutrient cycling, and carbon storage [9], with significant implications for plant growth and survival [7]. Among soil microbes, fungi are fundamental drivers of ecosystems processes owing to their role as decomposers, mutualists, or pathogens of plants and animals [10,11]. The role of soil fungi is, therefore, important for understanding the interplay between below- and above-ground processes and ecosystem functioning [12]. However, the effect of disturbances upon soil fungi has received less attention in comparison to above-ground responses (see: [13,14]), even if disturbances that alter drastically the soil components, and therefore soil fungal communities and networks, can have ecosystem-wide implications through their interactions and the biogeochemical process that they sustain [11,15]. For example, soil disruption produced by large-scale disturbances such as mines and linear infrastructures [16,17] causes important changes in soil and litter processes [10,18]. In these areas, disturbance-insensitive species or early successional plant species such as herbs are easily restored [4], while trees demand more time because of the abiotic and biotic stress conditions of the new environments [17]. In this context, recent findings suggest that soil microorganisms such as fungi are facilitators of the restoration and succession processes [19]. Previous studies have found that on mining spoils primary succession, root associated fungal communities and plant pathogens strongly influence plant community development [20,21]. In holm oak stands growing in metal contaminated soils, ectomycorrhizal fungal community composition and functional traits mediated plant performance and influenced plant capacity for phytoremediation of contaminants [22].

Moreover, plants can actively select their rhizosphere mycobiome [23,24]. For example, based on plant fitness and the mechanistic "C for nutrient trade" model [8], plants can discriminate between low-quality and high-quality partners to maintain nutrient uptake and plant vegetative growth [25,26]. In addition, plants 'functionally optimize' their symbiont community in particular conditions [27], provided that a wide soil fungal community is present. According to this theory, in degraded areas a lack of adequate soil fungal species composition and functioning might restrict the proper establishment and growth of tree species during the restoration of these areas.

Pine and oak species are among the most common trees used to restore forest areas in Spain [28]. However, it is well known that broadleaf and needle leaf species differ in photosynthetic rates, nutrient acquisition, nutrient economies, and root traits [29]. Recent works have demonstrated that differences in several traits (i.e. root morphology) between tree species affect root associated fungi mediated plant–soil feedbacks [30,31]. In this regard, in a mesh bag experiment in Spain, the seasonality in mycelial biomass was much lower for *Q. ilex* stands than in pine stands. This could suggest a greater mycorrhizal-based nutrient cycling in pines than in oak species in Mediterranean areas [32]. Similarly, in a litter decomposition study in pine and oak forests, suppression of mycorrhizal fungi accelerated litter decomposition in pine forests but had no effect in oak forests, suggesting that ecosystem function differences between plant communities are influenced by mycorrhizal community composition [29]. Thus, soil–plant–microbial interactions depend on the resident plants and fungal traits [33] and taxon-specific environmental requirements [10].

The aim of the study was to describe biomass and habitat-specific compositional differences of soil fungi to develop a preliminary theoretical framework for why the use of specific soil/litter inoculum might enhance tree seedlings' establishment success and growth. For this purpose, we described the soil fungal communities in four contrasting environments: (1) undisturbed native *Quercus robur* forest, (2) undisturbed *Pinus sylvestris* forest, (3) undisturbed *Quercus ilex* forest, and (4) a mining area that was naturally revegetated with shrubs and herbs. It must be considered that in large-scale disturbances such as mined sites, unfavorable abiotic and biotic condition [10] restricts the establishment and growth of tree seedlings [4]. Here, we assume that the differences in saprotrophic and mycorrhizal soil fungi between host species are related with their interaction with plants

to drive the best preferred/adequate nutrient economy (microbe-plant feedbacks). Thus, the use of a soil/litter inoculum from similar nearby forest areas upon planting or seeding can provide a satisfactory soil community over which to start the functional optimization dependent on the plant resources needed [26]. A preliminary description of soil–plant–microbial interactions of different species might help to design/develop more effective tree restoration strategies in Mediterranean degraded sites using soil/litter inoculum in planting/seeding to accelerate the man-induced tree seedling establishment process.

2. Materials and Methods

2.1. Study Sites and Design

In this work, we combined the datasets from two different set-ups, called "Forest" and "Mines" and located in Mediterranean Catalonia (North-eastern Spain, Figure 1). The climate in both sites is Mediterranean, with an average annual temperature of 12 °C and an annual rainfall of 600 mm, with summer droughts usually lasting for 2 months (July-August; [34]). The forest study consisted of six independent sites at an altitude of 1149 m a.s.l. (42°15′46.42″ N, 2°4′18.61″ E). Here, three monospecific forest stands of *Pinus sylvestris* (10 m × 10 m, n = 3) and three monospecific forest stands of *Quercus robur* (10 m × 10 m, n = 3) were randomly selected (Table 1). The mined study consisted of two independent stone quarries at an altitude of 300 m a.s.l., located in the Natural Area of PNIN-Poblet (Northeast Spain, 41°21′6.4728″ latitude N and 1°2′25.7496″ longitude E). In both quarries two areas were chosen: a *Quercus ilex* forest and a naturally revegetated plain area with shrubs and herbs, in each area three independent plots were established (total n = 12).

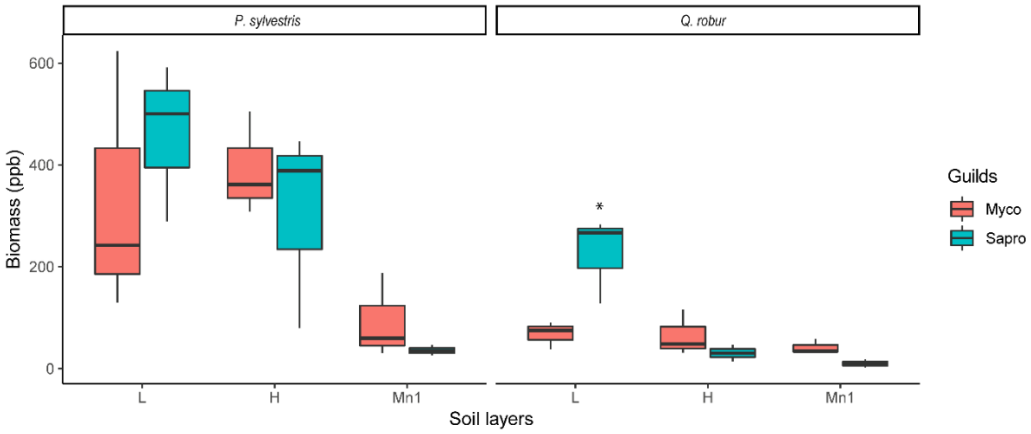

Figure 1. Boxplots of biomass of mycorrhizal and saprotrophic fungi (ppb) per three different soil layers (L = litter, H = humus, and Mn = mineral layer) for two different host species (*P. sylvestris* and *Q. robur*) in Mediterranean Spain. The asterisk over the boxplot indicates significant differences at $\alpha = 0.05$ between soil layers.

Table 1. Summary of the soil characteristics of the forest study: pH, organic matter (OM), Nitrogen (N) and phosphorus (P); n = 3: number of plots.

Forest Type (No. of Plots)	Range	pH	OM %	N %	P mg/kg
Ps	Min.	6.65	4.56	0.19	9.80
(n = 3)	Mean	7.38	11.12	0.5	17.07
	Max.	7.89	35.54	0.99	30.70
Qr	Min.	7.56	4.24	0.20	6.70
(n = 3)	Mean	7.69	9.14	0.38	10.05
	Max.	7.99	16.98	0.88	15.50

2.2. Soil Sampling

At each experiment, forest and mine, the soils were sampled during the Autumn season (October and November). Soil at the forest experiment was sampled in November 2017, and 4 soil samples were collected randomly in each of the 6 different stands with a drillable cylinder corer (diameter: 5 cm; depth: 12 cm; minimum distance between corers was 2 m). Each soil sample was split in 3 different horizons: litter (L): 0–5 cm, humus (H): 5–10 cm and mineral soil (Mn): 10–15 cm.

At the mine experiment, soil samples were collected in October 2019. In each mine, 3 *Quercus ilex* plots and 3 shrubland/grassland plots (named "plain"; >25 m distant from one another) were established at a minimum distance of 50 m between them. In each plot (10 m × 10 m), 4 randomly selected soil samples were collected with a drillable cylinder corer (diameter: 5 cm; depth: 12 cm; minimum distance between corers was 2 m). The shrub species were *Arbutus unedo, Viburnum thinus* and *Cistus salvifolius*. Each soil sample was split in 2 different horizons; humus (H) and mineral soil (Mn; total number of samples = 24).

In all experiments, the four soil subsamples were pooled in the field to obtain a composite sample per stand [11] and the mixed sample was placed on ice and taken to the laboratory. Afterwards, for each experiment each composite sample was sieved using 1 mm mesh and stored at 4 °C for less than 24 h until freeze-dried. Each sample was ground to fine powder using mortar and pestle to homogenize the soil core. The resulting pooled samples were stored at −20 °C before DNA extraction.

2.3. Fungal Community Analyses

Fungal DNA from the forest experiment was extracted from 150 mg of homogenized soil using the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol. Conversely, fungal DNA from the mine experiment was extracted from 500 mg of homogenized soil due to the higher mineral content in the soils.

For both experiments, we amplified the fungal internal transcribed spacer 2 (ITS2) region in a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) using the primers gITS7 [35] and ITS4 [35,36]. Each primer was fitted with 8-bp tags differing in at least three positions to individually identify each sample during posteriori bioinformatics analyses. We optimized the number of PCR cycles in each sample, aiming for weak to medium intensity PCR bands in the agarose gels, which was achieved in most of the samples by using 23–26 cycles. The final concentrations in the PCR reactions were: 12.5 ng template, 200 µM of each nucleotide, 2.75 mM $MgCl_2$, primers at 200 nM and 0.025 U μL^{-1} polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA, USA) in 1× buffer in 50 µL reactions. PCR cycling conditions were as follows: 5 min at 95 °C, followed by 23–26 cycles of 30 s at 95 °C, 30 s at 56 °C, 30 s at 72 °C and a final extension step at 72 °C for 7 min. Samples were amplified in triplicates together with negative controls employed during the DNA extraction and PCR, and the resulting PCR products were visualized on 1.5% agarose gel. Amplicons were purified using NGS clean-up and size selection kit (Macherey-Nagel, Duren, Germany), dissolved in 60 uL of elution buffer and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled, and the mix was purified using the EZNA Cycle Pure kit (Omega Bio-Tek) following manufacturer's protocol. Purified amplicons were quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA) and forest samples were sequenced at SciLifeLab NGI (Uppsala, Sweden) on a PacBio RS II system (Pacific Biosciences, Menlo Park, CA, USA) using four SMRT cells, while mine samples were sequenced at Centre of Genomic Regulation (Barcelona, Spain) on an Illumina MiSeq 2 × 300 bp. Finally, to estimate fungal biomass of the forest experiment free ergosterol was extracted and obtained in ppb (part per billion) as concentration of ergosterol per gram of sample [32].

2.4. Bioinformatic Analysis

Sequences were quality filtered and clustered using the SCATA pipeline (https://scata.mykopat.slu.se/ (accessed on 15 April 2021). We first removed DNA sequences with length <200, then the filtered sequences were screened for sample tags and primers defining a primer match of at least 90%. Sequences were pairwise compared using 'usearch' [37] after collapsing homopolymers to 3 bp. Sequences were quality filtered removing data with an amplicon quality score of <20 (averaged per sequence) and with a score of <10 at any position. Pairwise alignments were scored as follows: mismatch penalty of 1, gap open penalty of 0 and a gap extension penalty of 1. Putative chimera sequences were removed, and the quality-filtered sequences were clustered into species hypotheses [38] using single linkage clustering, with a maximum distance of 1.5% to the closest neighbour required to enter clusters. Global singletons were excluded from further analyses. Switched tags were detected when the two primers from the same sequence were found to have two distinct DNA tags and therefore these sequences were further excluded from the data. Sequence data are archived at NCBI's Sequence Read Archive under accession number PRJNA774050 (www.ncbi.nlm.nih.gov/sra, (accessed on 24 October 2021)).

2.5. Taxonomic and Functional Identification

We taxonomically identified the 1220 most abundant OTUs, which represented 93% of the total sequences in the two experiments. We selected the most abundant sequence from each OTU for taxonomic identification, using PROTAX software [39] implemented in PlutoF, using a 50% probability of correct classification (called by [39] as "plausible identifications"). These identifications were confirmed and some of them improved using massBLASTer in PlutoF against the UNITE [40]. Taxonomic identities at species level were assigned based on >98.5% similarity with database references, or to other lower levels using the next criteria: genus on >97%, family on >95%, order on >92% and phylum on >90% similarity. OTUs were assigned to the following functional guilds: (a) root-associated basidiomycetes, (b) root-associated ascomycetes, (c) moulds, (d) yeasts, (e) litter-associated basidiomycetes, (f) litter-associated ascomycetes, (g) pathogens, (h) moss-associated fungi, (i) soil saprotrophs (saprotrophic taxa commonly found in N-rich mineral soils), (j) unknown function, based on the UNITE database, DEEMY (www.deemy.de, (accessed on 5 May 2021)) or FUNGuild [41]. However, since most of the root-associated fungi were ectomycorrhizal species, for specific analyses, we used mycorrhizal community (which included root-associated basidiomycetes and root-associated ascomycetes) and saprotrophic fungal community (which included moss-associated fungi and soil saprotrophs).

2.6. Statistical Analyses

Statistical analyses were implemented in R software environment (version 4.0.3, R Development Core Team 2020), using the "ggplot2" package for figures [42] and the "nlme" package for linear mixed models (LMM, [43]) and the 'emmeans' package (v1.6.0, [44]) for post hoc pairwise comparisons between group means after LMMs.

As the experiments were sequenced using different methods, the analysis of each one was done independently. First, mycorrhizal and saprotrophs biomass, obtained in part per billions ergosterol per gram sample, was obtained by multiplying the total biomass by the relative abundance of each functional guild (Table S1). The differences in biomass of the fungal groups (i.e., saprotroph and mycorrhizal) per each host and revegetated plain area were analysed using LMM. In forest experiments, the random factor was sample identity, since different depths were collected from same site (site dependency), while in the mined experiment the random factor was mine identity nested with plots, since samples coming from plots from the same mine are more similar (site and spatial dependency).

3. Results

Overall, the forest monitoring showed that the most abundant phylum in both pine and oak stands was Ascomycota (54.30% ± 5.9% of the sequences) followed by Basidiomycota (45.71% ± 5.9%). The most abundant guilds in pine were root-associated basidiomycetes and moulds, representing 44.6% ± 6.8% and 25.8% ± 6.9% of the sequences, respectively, followed by saprotrophs (12.2% ± 2.2%). The most abundant guilds in oak were root-associated basidiomycetes and saprotrophs with 48.6% ± 6.3% and 23.6% ± 5.9% sequence read proportions. Similarly, the mine experiment showed that the most abundant phylum in both forest and revegetated plain area was Ascomycota (85.19% ± 3.27% and 63.99% ± 4.13% of the sequences) followed by Basidiomycota (14.06% ± 3.32% and 35.85% ± 4.16% of the sequences). The most abundant guilds in the holm oak forest were saprotrophs, representing 67.54% ± 12.53%, followed by root associated fungi with 32.46% ± 5.64% of the sequences. Conversely, the most abundant guild in the revegetated plain was root associated ascomycetes, representing 17.0% ± 2.4% of the sequences, followed by root associated basidiomycetes, (12.2% ± 2.2%). For the downstream analyses, we used mycorrhizal community (which included root-associated basidiomycetes and root-associated ascomycetes).

3.1. Soil Fungi Differences between Pine and Oak Stands (Forest Experiment)

The forest study showed clearly that fungal biomass distribution among soil layers differed between pine and oak stands. In pine stands, there were no significant interactive effects among saprotrophs and mycorrhizal biomass between soil layers (Fungal type×depth interaction $F_{[2,6]} = 1.49$, p-value = 0.298; Figure 1). However, there was a significant depth effect ($F_{[2,6]} = 5.45$, p-value = 0.044; Figure 1), with greater biomass of saprotrophic and mycorrhizal fungi in L and H layers compared with mineral soil. In contrast, in oak stands, there was a significant interactive effect among biomass of saprotrophic and mycorrhizal biomass between soil layers (Fungal type × depth interaction $F_{[2,6]} = 20.20$, p-value = 0.002; Figure 1), with greater biomass of saprotrophic fungi than mycorrhizal fungi in litter layer (L). Conversely, no biomass differences were found in the humus (post-hoc paired t-ratio = 1.5, p-value = 0.676) and mineral layers between mycorrhizal fungi and saprotrophic fungi (post-hoc paired t-ratio = 1.23, p-value = 0.810).

Mycorrhizal fungi dominated the humus and mineral soil fungal communities in both natural systems (Figure 1). However, in the oak forest litter fungal community was dominated by saprotrophs, with *Trichoderma*, *Leohumicola*, *Preussia*, and *Tetracladium* as most abundant genera (Figure 2d). In pine stands, litter was equally dominated by mycorrhizal generalists such as *Tomentella*, *Inocybe* and *Tricholoma* and saprotrophs with genera such as *Leohumicola*, *Trichoderma* and *Tetracladium* (Figure 2a). In pine stands the most abundant genera in humus and mineral soil fungal communities were also *Tomentella*, *Tricholoma*, *Inocybe*, *Lactarius* and *Rhizopogon*; in contrast, *Cortinarius*, *Sebacina*, and *Russula* were the dominant fungal genera in oak stands (Figure 2b). Interestingly, the pine saprotrophs were more abundant in the mineral layer with *Leohumicola*, *Cadophora*, *Tetracladium* as the most abundant genera (Figure 2c).

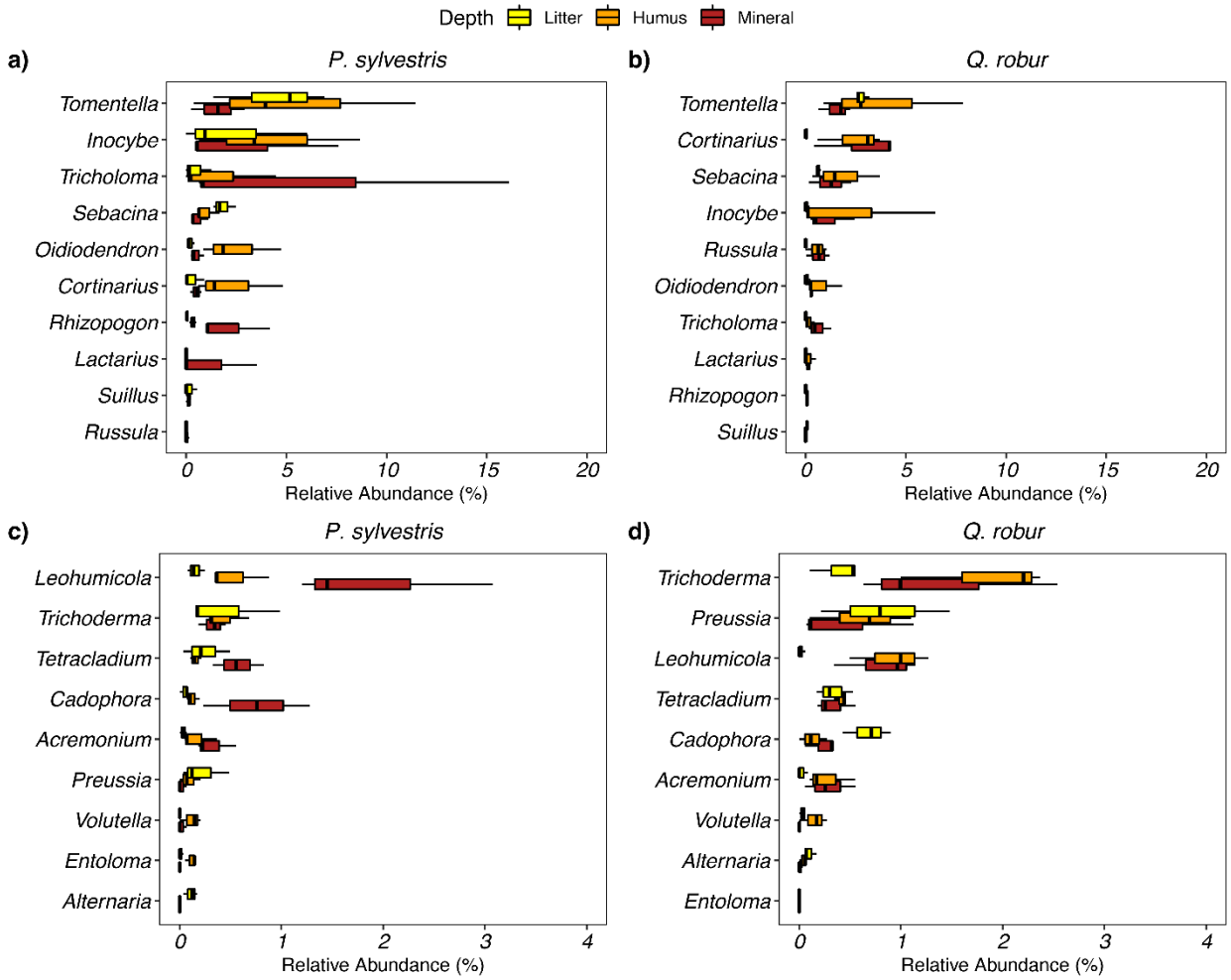

Figure 2. Boxplots of relative read abundance (%) of (**a**,**b**) mycorrhizal and (**c**,**d**) saprotrophic fungi for the most abundant species in pine and oak soil layers (litter-L, humus-H and mineral-Mn).

3.2. Soil Fungi Differences between Q. ilex and Revegetated Quarry (Mine Experiment)

The mine experiment showed that fungal biomass among soil layers differed between the holm oak and plain areas. The oak forest was dominated by saprotrophs and plain areas by mycorrhizal species. In the oak forest, there was no significant fungi-depth interaction among saprotrophs and mycorrhizal fungi (Fungal type × depth interaction $F_{[1,5]} = 0.42$, p-value = 0.528; Figure 3). Here, the saprotrophs occurred in both layers (H and Mn), while mycorrhizal fungi showed significantly greater presence in mineral soil than in humus layer (Figure 4a). In contrast, in plain areas dominated by shrubs/herbs, fungi-depth interaction (Fungal type×depth interaction $F_{[1,5]} = 0.39$, p-value = 0.548; Figure 3) was opposite, with greater biomass of saprotrophs than mycorrhizal fungi in humus layer (H), while no differences were found between mycorrhizal species among H and Mn soil layers (post-hoc paired t-ratio = 0.70, p-value = 0.892).

EM fungi dominated the humus and mineral soil fungal communities in revegetated plain area (Figure 3), while in forest system saprotrophs dominated both soil layers (H and Mn). In holm oak forest, humus and mineral layers were dominated by mycorrhizal genera, *Oidiodendron* followed by *Inocybe* and *Tuber* (Figure 4a). In plain revegetated areas, the mycorrhizal genus *Oidiodendron* was again the most abundant, followed *Russula*, *Tomentella*, *Inocybe* and *Sebacina* genera (Figure 4b). Interestingly, clear compositional differences were found for saprotrophic genera between forest and plain sites, with *Alternaria*, *Bovista*, *Mycena* and *Agaricus* dominating the soil humus forest layer, while the saprotrophic genus *Mycena* clearly dominated the revegetated plain area. (Figure 4c,d). In mineral layer,

soil forest communities were dominated by saprotrophic genera such as *Alternaria* and *Cadophora*, while in the revegetated plain area, *Chalara*, *Cadophora* and *Mycena* were the dominant saprotrophic genera (Figure 4d).

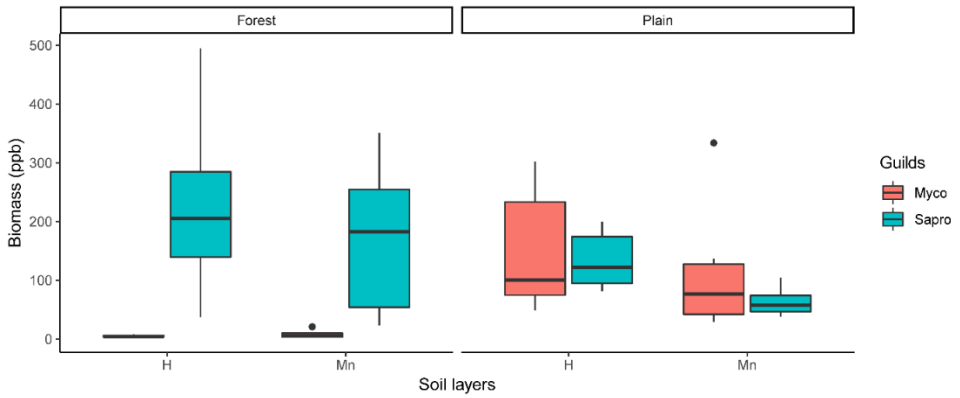

Figure 3. Boxplots of biomass of mycorrhizal and saprotrophic fungi (ppb) per two different soil layers (H = humus, and Mn = mineral layer) in two different areas in the mine experiment (forest = *Q. ilex* stand, and Plain = shrubs/grassland area) in Mediterranean Spain.

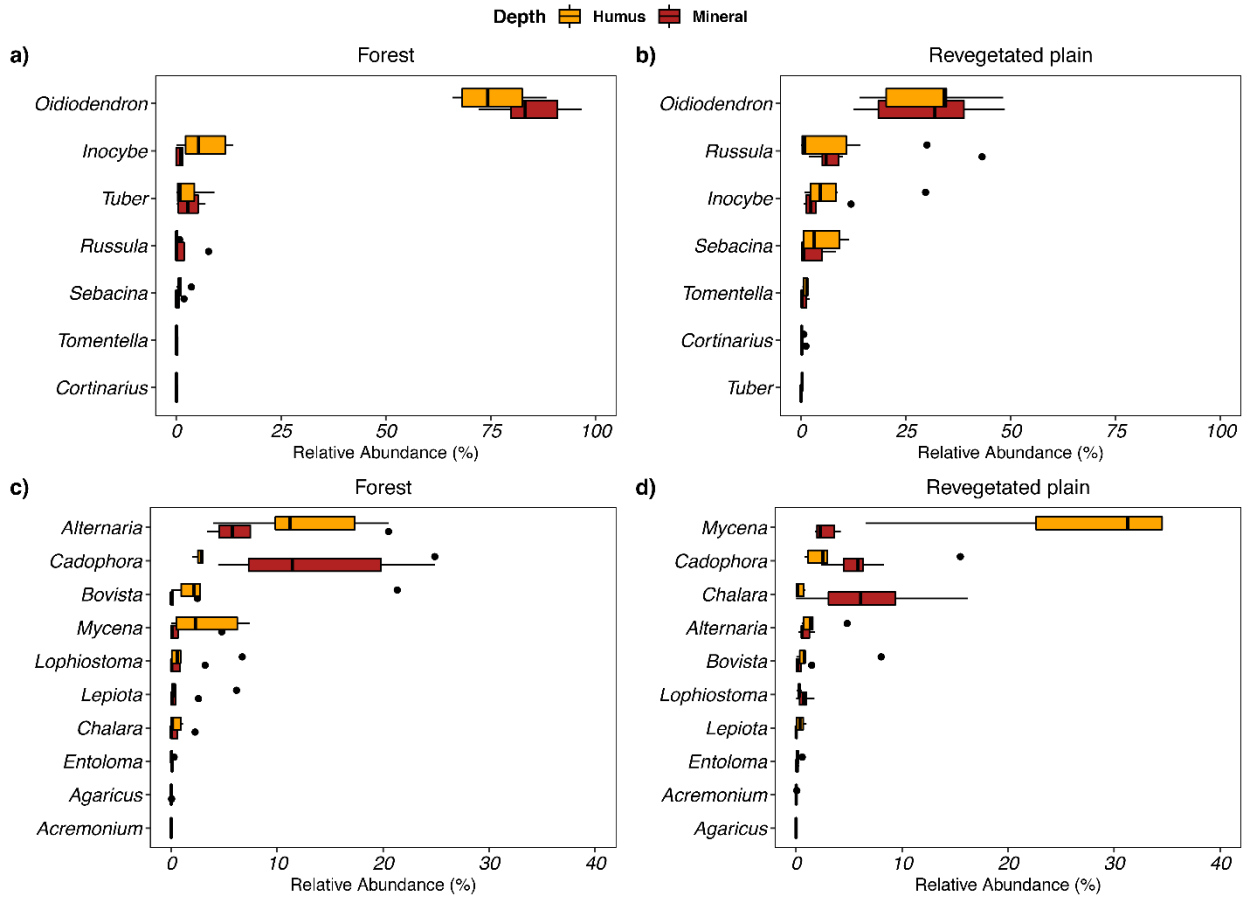

Figure 4. Boxplots of relative read abundance (%) of the most abundant (**a**,**b**) mycorrhizal fungi and (**c**,**d**) saprotrophic fungi in the holm oak forest and revegetated plain soil layers (humus-H and mineral layers-Mn).

4. Discussion

The central finding of this study is that soil fungal biomass and composition differed between the four environments selected. The differences were significant between the pine

and oak forests, and in the mined sites between holm oak forest and revegetated plain with shrubs and herbs. At the same time, soil fungal communities varied within a habitat between litter, humus and mineral soil layers, especially in pine and oak forest.

4.1. Soil Fungi Differences between Pine and Oak Stands (Forest Study)

As expected, soil mycorrhizal biomass dominated pine stands, which is in accordance with [45] who described in Mediterranean pine stands a dominance of mycorrhizal biomass compared to other fungi guilds in soil mineral layers. Although with low biomass, the mycorrhizal dominance in mineral layers has been explained by the need of pines to establish a good net of mycorrhizal species to mine for nutrients [46]. However, in pine stands the biomass values of saprotrophic fungi in litter and humus layers was similar to that of mycorrhizal fungi. Also, for both guilds the highest biomass values were observed in litter and humus layers. The biomass estimates of saprotrophic fungi in litter and humus layers were similar to those of mycorrhizal fungi suggesting a saprotrophic-mycorrhizal fungal competition in these layers for nutrients, which is related to the Gadgil effect [47]. It seems that when more recalcitrant litter, such as needles, and a great mycorrhizal biomass were concurrent, the nitrogen uptake by mycorrhizal species slows the decomposition process by saprotrophs as a consequence [29]. This process results in an accumulation of organic matter in soil with a significant fraction of organic nutrients that can be used by mycorrhizal fungi to acquire nitrogen and phosphorus [8]. This gives a major role to mycorrhizal fungi in the nutrient cycling in pine stands. Interestingly, in pine stands the mycorrhizal genera that dominate the three soil layers are *Tomentella*, *Inocybe* and *Tricholoma*. Our results showed a clear dominance of mycorrhizal species in the humus and litter layers for pine. In this regard, previous studies have shown that different mycorrhizal species can disrupt organic matter and liberate N via oxidative decomposition by expressing of set of transcriptomes encoding proteins associated with oxidation of lignocellulose by saprotrophic fungi [48,49]. From a restoration point of view this may indicate that in the pine planting/seeding, actions targeted to restore highly degraded ecosystems, the use of a small amount of soil, litter or both from nearby pine stands as inoculum to add most common mycorrhizal species may be beneficial (Figure 5). The inclusion of soil or litter inoculum from undisturbed areas at the pine tree planting or seeding phase can provide more varied soil fungal community over which to start the functional optimization based on resources needed by each seedling.

The oak stands of the forest study showed a contrary pattern compared to pines, with saprotrophic fungi having low biomass in humus and mineral soil but clearly dominating the leaf litter layer, which is more nutrient rich than a needle litter layer [50]. In contrast, the mycorrhizal species in oak stands showed stable biomass values around 100 ppb in the three soil layers, which was significantly lower than that observed for mycorrhizal species in pine stands. These differences in mycorrhizal biomass were particularly significant in litter and humus layers. Our results are in accordance with [32] who quantified the mycelial mycorrhizal biomass in different Mediterranean pine and oak forests, the data showing that mycorrhizal biomass was three-fold greater in pine forests than in oak stands. It seems that in oak forests saprotrophs are the main fungi responsible for decoupling C:N from litter, in contrast to pine stands, where mycorrhizal fungi may influence litter decomposition as well [51]. Thus, it seems that in Mediterranean oak stands the fast decomposition of high-quality litter pools by saprotrophs might cause higher C and N mineralization that can be then mined by mycorrhizal species in the mineral soil [8]. In any case, the dominant mycorrhizal genera in oak stands, *Tomentella*, *Cortinarius*, *Sebacina* and *Inocybe,* were mainly located in humus and mineral soil layers, with low presence in the litter layer. In contrast, the dominant genera of saprotrophic fungi (i.e., *Preussia*, *Tetracladium*) in soil were well distributed across three soil layers. From a restoration point of view, when the purpose is to restore a highly degraded area by planting or seeding of oak species, it might be advantageous to use humus or mineral soil as inoculum from nearby undisturbed oak

stands to provide a starting soil fungal community adapted to oaks over which to start the functional optimisation depending on oak seedling requirements (Figure 5).

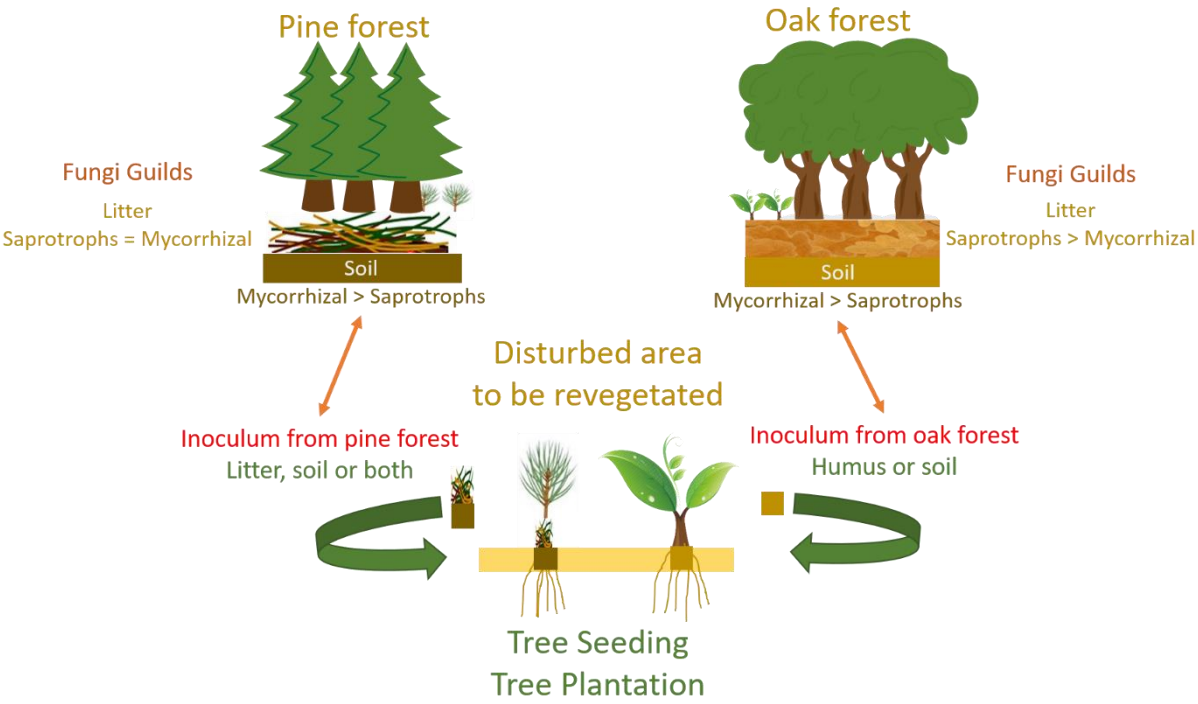

Figure 5. Summary of the differences in soil fungi guilds between pine and oak forest considering three soil layers. The possible types of inoculums to be used in restoration frameworks that maintain the most important fungi for pine and oak trees are shown. Litter, soil or mixed inoculum is recommended for pine and use of soil or humus is recommended for oak. Adequate inoculum might accelerate the man-induced tree seedling establishment process.

4.2. Soil Fungi Differences between Q. ilex and Revegetated Quarry (Mine Study)

In mined sites, the overall results followed the main fungal differences described in forest study when holm oak plots and plain area are compared. The holm oak forest had both soil layers (humus and mineral) dominated by saprotrophic fungi, although the main differences between mycorrhizal fungi and saprotrophs were largely in the soil humus layer (Figure 3). The pattern of greater saprotroph biomass is related with the rate of litter decomposition [52], as it may accelerate the mineralisation of decomposed organic leaf nutrients, as discussed above. Thus, in Mediterranean Spain saprotrophic fungi play a fundamental role in nutrient cycling in stands of both the deciduous *Q. robur* and the evergreen *Q. ilex*, species that differ also in leaf texture [53]. From the compositional perspective, in oak stands the mycorrhizal genus *Oidiodendron* dominated both humus and mineral soil, while the community of saprotrophic fungi was more diverse in both soil layers, with *Cadophora* and *Chalara* dominating. Thus, these results also suggest that the use of humus or mineral soil as inoculum might be an option to provide a wide fungal community to facilitate seedling establishment (Figure 5).

Finally, the plain natural revegetated areas were dominated by mycorrhizal species in both soil layers, which is possibly related with the shrub species such as *Rosa* spp., *Crataegus monogyna* and *Thymus mastichina* that colonised these areas over time [54], while saprotrophic fungi were more abundant in the humus layer. Interestingly, from a compositional perspective there were differences in soil fungi genera between the holm oak forest and plain area even if the sites were in close proximity. These differences might be produced by the different successional stage of the habitats [55], of which holm oak forest had more stable plant and soil fungal communities, while early successional mined areas, such as our plain sites, have a more shifting plant system and rhizosphere mycobiome [56]. Thus, the

soil fungal composition of these plain sites might not be suitable for tree establishment and growth of advanced successional tree species considering the interplay between plants and fungi [57]. Finally, the comparison of fungal biomass values between different functional groups requires caution since the ergosterol to biomass conversion may vary among fungal species, structures, and growth conditions [58]. However, our results agree with previously studies where fungal biomass was assessed between different forest types [32].

5. Conclusions

Our analyses indicate that soil fungal biomass and composition differed between the four environments selected, with significant differences especially between pine and oak forest, and in mined sites between holm oak forest and revegetated plain. Moreover, soil fungal communities differed within environments between litter, humus and mineral soil layers, suggesting that different soil/litter inoculum may be needed for restoration of different tree species. For example, the dominance of mycorrhizal species in the humus and litter layers in pine stands suggest that one could use a small amount of soil, litter or both from nearby pine stands as inoculum to introduce the most common mycorrhizal species in highly degraded ecosystems. Conversely, in pure oak stands characterized by the dominance of mycorrhizal fungi in humus and mineral layers, the use of humus or mineral soil from nearby undisturbed oak stands as inoculum could provide a starting soil fungal community adapted to oaks. It seems that in each environment the soil–plant–microbial interactions are different depending on the soil layer selected. Finally, further research is needed to test the effectiveness of the proposed preliminary framework and to disentangle the drivers of successful forest restoration in Mediterranean ecosystems.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f13010088/s1. Table S1: Fungal biomass values for the forest and mine experiment. Fungal biomass was obtained multiplying the total biomass by the relative abundance of each functional guild. Ps = *P. sylvestris*, Qr = *Q. robur*.

Author Contributions: Conceptualization, I.A. and J.G.A.; methodology, I.A., S.D. and J.G.A.; software, I.A. and J.G.A.; validation, I.A. and J.G.A.; formal analysis, I.A. and J.G.A.; investigation, I.A. and J.G.A.; resources, J.G.A.; data curation, I.A., S.D. and J.G.A.; writing—original draft preparation, I.A. and J.G.A.; writing—review and editing, I.A., SD and J.G.A.; visualization, I.A., S.D. and J.G.A.; supervision, J.G.A.; project administration, J.G.A.; funding acquisition, J.G.A. All authors have read and agreed to the published version of the manuscript.

Funding: This project has received funding from the European Union's H2020 research and innovation programme under Marie Sklodowska-Curie grant agreement No 801586. This work was supported by the Spanish Ministry of Science, Innovation and Universities, grants RTI2018-099315-A-I00. I.A. was supported by a H2020-Marie Slodowska Curie Action Cofund fellowship (801596) and J.G.A. was supported by Ramon y Cajal fellowship (RYC-2016-20528).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be made available upon reasonable request to the first author.

Acknowledgments: We thank Carles Castaño for assistance in data preparation.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Watson, J.E.M.; Evans, T.; Venter, O.; Williams, B.; Tulloch, A.; Stewart, C.; Thompson, I.; Ray, J.C.; Murray, K.; Salazar, A.; et al. The exceptional value of intact forest ecosystems. *Nat. Ecol. Evol.* **2018**, *2*, 599–610. [CrossRef] [PubMed]
2. Paustian, K.; Lehmann, J.; Ogle, S.; Reay, D.; Robertson, G.P.; Smith, P. Climate-smart soils. *Nat. Cell Biol.* **2016**, *532*, 49–57. [CrossRef] [PubMed]
3. Sharma, S.; MacKenzie, R.A.; Tieng, T.; Soben, K.; Tulyasuwan, N.; Resanond, A.; Blate, G.; Litton, C.M. The impacts of degradation, deforestation and restoration on mangrove ecosystem carbon stocks across Cambodia. *Sci. Total. Environ.* **2020**, *706*, 135416. [CrossRef] [PubMed]

4. Alday, J.G.; Zaldívar, P.; Torroba-Balmori, P.; Fernández-Santos, B.; Martínez-Ruiz, C. Natural forest expansion on re-claimed coal mines in Northern Spain: The role of native shrubs as suitable microsites. *Environ. Sci. Pollut. Res.* **2016**, *23*, 13606–13616. [CrossRef]
5. Brooker, R.W.; Maestre, F.T.; Callaway, R.M.; Lortie, C.L.; Cavieres, L.A.; Kunstler, G.; Liancourt, P.; Tielborger, K.; Travis, J.M.J.; Anthel-me, F.; et al. Facilitation in plant communities: The past, the present, and the future. *J. Ecol.* **2008**, *96*, 18–34. [CrossRef]
6. Onaindia, M.; Ametzaga, I.; San Sebastián, M.; Mitxelena, A.; Rodríguez-Loinaz, G.; Peña, L.; Alday, J.G. Can understorey na-tive woodland plant species regenerate under exotic pine plantations using natural succession? *For. Ecol. Man-Agement* **2013**, *308*, 136–144. [CrossRef]
7. van der Putten, W.H.; Bardgett, R.D.; Bever, J.D.; Martijn Bezemer, T.; Casper, B.B.; Fukami, T.; Kardol, P.; Klironomos, J.N.; Kulmatiski, A.; Schweitzer, J.A.; et al. Plant-soil feed-backs: The past, the present and future challenges. *J. Ecol.* **2013**, *101*, 265–276. [CrossRef]
8. Phillips, R.P.; Brzostek, E.; Midgley, M.G. The mycorrhizal-associated nutrient economy: A new framework for predicting carbon–nutrient couplings in temperate forests. *New Phytol.* **2013**, *199*, 41–51. [CrossRef] [PubMed]
9. Bardgett, R.D.; van der Putten, W. Belowground biodiversity and ecosystem functioning. *Nat. Cell Biol.* **2014**, *515*, 505–511. [CrossRef]
10. Tedersoo, L.; Bahram, M.; Põlme, S.; Kõljalg, U.; Yorou, N.S.; Wijesundera, R.L.C.; Ruiz, L.V.; Vasco-Palacios, A.M.; Thu, P.Q.; Suija, A.; et al. Global diversity and geography of soil fungi. *Science* **2014**, *346*, 1256688. [CrossRef]
11. Adamo, I.; Piñuela, Y.; Bonet, J.A.; Castaño, C.; de Aragón, J.M.; Parladé, J.; Pera, J.; Alday, J.G. Sampling forest soils to describe fungal diversity and composition. Which is the optimal sampling size in mediterranean pure and mixed pine oak forests? *Fungal Biol.* **2021**, *125*, 469–476. [CrossRef]
12. Urbina, I.; Grau, O.; Sardans, J.; Ninot, J.M.; Peñuelas, J. Encroachment of shrubs into subalpine grasslands in the Pyrenees changes the plant-soil stoichiometry spectrum. *Plant Soil* **2020**, *448*, 37–53. [CrossRef]
13. Lourenço, K.S.; Suleiman, A.K.A.; Pijl, A.; Cantarella, H.; Kuramae, E.E. Dynamics and resilience of soil myco-biome under multiple organic and inorganic pulse disturbances. *Sci. Total Environ.* **2020**, *733*, 139173. [CrossRef]
14. Rodriguez-Ramos, J.C.; Cale, J.A.; Cahill Jr, J.F.; Simard, S.W.; Karst, J.; Erbilgin, N. Changes in soil fungal community composition depend on functional group and forest disturbance type. *New Phytol.* **2021**, *229*, 1105–1117. [CrossRef] [PubMed]
15. Barnes, A.D.; Allen, K.; Kreft, H.; Corre, M.D.; Jochum, M.; Veldkamp, E.; Clough, Y.; Daniel, R.; Darras, K.; Denmead, L.H.; et al. Direct and cascading impacts of tropi-cal land-use change on multi-trophic biodiversity. *Nat. Ecol. Evol.* **2017**, *1*, 1511–1519. [CrossRef]
16. Alday, J.G.; Marrs, R.H.; Martínez-Ruiz, C. Vegetation succession on reclaimed coal wastes in Spain: The influence of soil and environmental factors. *Appl. Veg. Sci.* **2010**, *14*, 84–94. [CrossRef]
17. Alday, J.G.; Marrs, R.H.; Martínez-Ruiz, C. Vegetation convergence during early succession on coal wastes: A 6-year permanent plot study. *J. Veg. Sci.* **2011**, *22*, 1072–1083. [CrossRef]
18. Zhou, L.; Li, H.; Shen, H.; Xu, Y.; Wang, Y.; Xing, A.; Fang, J. Shrub-encroachment induced alterations in input chemistry and soil microbial community affect topsoil organic carbon in an Inner Mongolian grassland. *Biogeochemistry* **2017**, *136*, 311–324. [CrossRef]
19. Qiang, W.; He, L.; Zhang, Y.; Liu, B.; Liu, Y.; Liu, Q.; Pang, X. Aboveground vegetation and soil physicochemical properties jointly drive the shift of soil microbial community during subalpine secondary succession in southwest China. *Catena* **2021**, *202*, 105251. [CrossRef]
20. Krüger, C.; Kohout, P.; Janoušková, M.; Püschel, D.; Frouz, J.; Rydlová, J. Plant communities rather than soil prop-erties structure arbuscular mycorrhizal fungal communities along primary succession on a mine spoil. *Front. Micro-Biol.* **2017**, *8*, 719. [CrossRef] [PubMed]
21. Kolaříková, Z.; Kohout, P.; Krüger, C.; Janoušková, M.; Mrnka, L.; Rydlová, J. Root-associated fungal communities along a primary succession on a mine spoil: Distinct ecological guilds assemble differently. *Soil Biol. Biochem.* **2017**, *113*, 143–152. [CrossRef]
22. Gil-Martínez, M.; López-García, Á.; Dominguez, M.T.; Navarro-Fernández, C.M.; Kjøller, R.; Tibbett, M.; Marañón, T. Ectomycorrhizal Fungal Communities and Their Functional Traits Mediate Plant–Soil Interactions in Trace Element Contaminated Soils. *Front. Plant Sci.* **2018**, *9*, 1682. [CrossRef]
23. Berendse, F.; Jonasson, S. Nutrient Use and Nutrient Cycling in Northern Ecosystems. In *Arctic Ecosystems in a Changing Climate*; Elsevier: Alpharetta, GA, USA, 1992; pp. 337–356.
24. Tkacz, A.; Cheema, J.; Chandra, G.; Grant, A.; Poole, P.S. Stability and succession of the rhizosphere microbiota depends upon plant type and soil composition. *ISME J.* **2015**, *9*, 2349–2359. [CrossRef]
25. Bogar, L.; Peay, K.; Kornfeld, A.; Huggins, J.; Hortal, S.; Anderson, I.; Kennedy, P. Plant-mediated partner discrim-ination in ectomycorrhizal mutualisms. *Mycorrhiza* **2019**, *29*, 97–111. [CrossRef]
26. Blažková, A.; Jansa, J.; Püschel, D.; Vosátka, M.; Janoušková, M. Is mycorrhiza functioning influenced by the quan-titative composition of the mycorrhizal fungal community? *Soil Biol. Biochem.* **2021**, *157*, 108249. [CrossRef]
27. Werner, G.D.A.; Zhou, Y.; Pieterse, C.M.J.; Kiers, E.T. Tracking plant preference for higher-quality mycorrhizal sym-bionts under varying CO2 conditions over multiple generations. *Ecol. Evol.* **2018**, *8*, 78–87. [CrossRef] [PubMed]

28. Quinto, L.; Navarro-Cerrillo, R.M.; Palacios-Rodriguez, G.; Ruiz-Gómez, F.; Duque-Lazo, J. The current situation and future perspectives of *Quercus ilex* and *Pinus halepensis* afforestation on agricultural land in Spain under climate change scenarios. *New For.* **2021**, *52*, 145–166. [CrossRef]
29. Fernandez, C.W.; See, C.R.; Kennedy, P.G. Decelerated carbon cycling by ectomycorrhizal fungi is controlled by substrate quality and community composition. *New Phytol.* **2020**, *226*, 569–582. [CrossRef] [PubMed]
30. Teste, F.P.; Kardol, P.; Truner, B.L.; Wardle, D.A.; Zemunik, G.; Renton, M.; Laliberté, E. Plant-soil feedback and the maintainence of diversity in Mediterranean-climate shrublands. *Science* **2017**, *355*, 173–176. [CrossRef] [PubMed]
31. Segnitz, R.M.; Russo, S.E.; Davies, S.J.; Peay, K.G. Ectomycorrhizal fungi drive positive phylogenetic plant–soil feedbacks in a regionally dominant tropical plant family. *Ecology* **2020**, *101*, e03083. [CrossRef]
32. Hagenbo, A.; Piñuela, Y.; Castaño, C.; de Aragón, J.M.; de-Miguel, S.; Alday, J.G.; Bonet, J.A. Production and turnover of mycorrhizal soil mycelium relate to variation in drought conditions in Mediterranean *Pinus pinaster, Pinus sylvestris* and *Quercus ilex* forests. *New Phytol.* **2021**, *230*, 1609–1622. [CrossRef] [PubMed]
33. Smith, G.R.; Peay, K.G. Stepping forward from relevance in mycorrhizal ecology. *New Phytol.* **2020**, *226*, 292–294. [CrossRef] [PubMed]
34. Alday, J.G.; De Aragón, J.M.; de-Miguel, S.; Bonet, J.A. Mushroom biomass and diversity are driven by different spatio-temporal scales along Mediterranean elevation gradients. *Sci. Rep.* **2017**, *7*, 1–11.
35. Ihrmark, K.; Bödeker, I.T.; Cruz-Martinez, K.; Friberg, H.; Kubartova, A.; Schenck, J.; Strid, Y.; Stenlid, J.; Brandström-Durling, M.; Clemmensen, K.; et al. New primers to amplify the fungal ITS2 region—Evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiol. Ecol.* **2012**, *82*, 666–677. [CrossRef] [PubMed]
36. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc. *Guide Methods Appl.* **1990**, *18*, 315–322.
37. Edgar, R.C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **2010**, *26*, 2460–2461. [CrossRef]
38. Kõljalg, U.; Nilsson, R.H.; Abarenkov, K.; Tedersoo, L.; Taylor, A.F.; Bahram, M.; Larsson, K.H. Towards a uni-fied paradigm for sequence-based identification of fungi. *Wiley Online Libr.* **2013**, *22*, 5271–5277.
39. Somervuo, P.; Koskela, S.; Pennanen, J.; Nilsson, R.H.; Ovaskainen, O. Unbiased probabilistic taxonomic classification for DNA barcoding. *Bioinformatics* **2016**, *32*, 2920–2927. [CrossRef]
40. Abarenkov, K.; Nilsson, R.H.; Larsson, K.-H.; Alexander, I.J.; Eberhardt, U.; Erland, S.; Høiland, K.; Kjøller, R.; Larsson, E.; Pennanen, T.; et al. The UNITE database for molecular identification of fungi–recent updates and future perspectives. *New Phytol.* **2010**, *186*, 281–285. [CrossRef]
41. Nguyen, N.H.; Song, Z.; Bates, S.T.; Branco, S.; Tedersoo, L.; Menke, J.; Schilling, J.S.; Kennedy, P.G. FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecol.* **2016**, *20*, 241–248. [CrossRef]
42. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2016; ISBN 978-3-319-24277-4.
43. Pinheiro, J.C.; Bates, D.M.; DebRoy, S.; Sakar, D. The Nlme Package: Linear and nonlinear mixed efects models, R Version 3. 2012. Available online: https://svn.r-project/R-packages/trunk/nlme. (accessed on 7 September 2021).
44. Lenth, R. Estimated Marginal Means, Aka Least-Squares Means, 1.4.8; R Package. Available online: https://CRAN.R-project.org/package=emmeans (accessed on 5 June 2021).
45. Castaño, C.; Alday, J.G.; Lindahl, B.D.; de Aragón, J.M.; de-Miguel, S.; Colinas, C.; Bonet, J.A. Lack of thinning effects over inter-annual changes in soil fungal community and diversity in a Mediterranean pine forest. *For. Ecol. Manag.* **2018**, *424*, 420–427. [CrossRef]
46. Querejeta, J.I.; Roldán, A.; Albaladejo, J.; Castillo, V. The role of mycorrhizae, site preparation, and organic amendment in the afforestation of a semi-arid Mediterranean site with *Pinus halepensis*. *For. Sci.* **1998**, *44*, 203–211.
47. Fernandez, C.W.; Kennedy, P.G. Revisiting the 'Gadgil effect': Do interguild fungal interactions control carbon cycling in forest soils? *New Phytol.* **2016**, *209*, 1382–1394. [CrossRef]
48. Shah, F.; Nicolás, C.; Bentzer, J.; Ellström, M.; Smits, M.; Rineau, F.; Canbäck, B.; Floudas, D.; Carleer, R.; Lackner, G. Ectomycorrhizal fungi decompose soil organic matter using oxidative mechanisms adapted from saprotrophic ancestors. *New Phytol.* **2016**, *209*, 1705–1719. [CrossRef]
49. Nicolás, C.; Martin-Bertelsen, T.; Floudas, D.; Bentzer, J.; Smits, M.; Johansonn, T.; Troein, C.; Persson, P.; Tulind, A. The soil organic matter decomposition mechanisms in ectomycorrhizal fungi are tuned for liberating soil organic nitrogen. *ISME J.* **2019**, *13*, 977–988. [CrossRef]
50. Berg, B.; Meentemeyer, V. Litter quality in a north European transect versus carbon storage potential. *Plant Soil* **2002**, *242*, 83–92. [CrossRef]
51. Voříšková, J.; Brabcová, V.; Cajthaml, T.; Baldrian, P. Seasonal dynamics of fungal communities in a temperate oak forest soil. *New Phytol.* **2014**, *201*, 269–278. [CrossRef]
52. Guénon, R.; Day, T.A.; Velazco-Ayuso, S.; Gros, R. Mixing of Aleppo pine and Holm oak litter increases biochem-ical diversity and alleviates N limitations of microbial activity. *Soil Biol. Biochem.* **2017**, *105*, 216–226. [CrossRef]
53. Terradas, J. *Holm Oak and Holm Oak Forests: An Introduction. En Ecology of Mediterranean Evergreen Oak Forests*; Springer: Berlin/Heidelberg, Germany, 1999; pp. 3–14.
54. Allen, E.B.; Allen, M.F. Competition between plants of different successional stages: Mycorrhizae as regulators. *Can. J. Bot.* **1984**, *62*, 2625–2629. [CrossRef]

55. Liu, L.; Zhu, K.; Krause, S.M.; Li, S.; Wang, X.; Zhang, Z.; Zhang, J. Changes in assembly processes of soil micro-bial communities during secondary succession in two subtropical forests. *Soil Biol. Biochem.* **2021**, *154*, 108144. [CrossRef]
56. Harantová, L.; Mudrák, O.; Kohout, P.; Elhottová, D.; Frouz, J.; Baldrian, P. Development of microbial community during primary succession in areas degraded by mining activities. *Land Degrad. Dev.* **2017**, *28*, 2574–2584. [CrossRef]
57. Urbanová, M.; Šnajdr, J.; Baldrian, P. Composition of fungal and bacterial communities in forest litter and soil is largely determined by dominant trees. *Soil Biol. Biochem.* **2015**, *84*, 53–64. [CrossRef]
58. Wallander, H.; Ekblad, A.; Godbold, D.L.; Johnson, D.; Bahr, A.; Baldrian, P.; Björkf, R.G.; Kieliszewska-Rokickag, B.; Kjøllerh, R.; Kraigher, H.; et al. Evaluation of methods to estimate production, biomass and turnover of ectomycorrhizal mycelium in forests soils–A review. *Soil Biol. Biochem.* **2013**, *57*, 1034–1047. [CrossRef]