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RESEARCH ARTICLE

Short-term thinning effects on saprotrophic and ectomycorrhizal soil fungal communities in a *Pinus halepensis* common garden

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Abstract

Background and aim Soil fungal communities can vary in their abundance and diversity between host tree species, but also between genotypes within the same host tree species. However, there are conflicting results on the role of host tree genetics in shaping soil fungal communities and how silvicultural treatments can influence their dynamics.

Methods We investigated whether genetic variation among 20 populations representing five ecotypes of *Pinus halepensis,* the most widespread tree species in the Mediterranean basin, affects their soil fungal community, before and after a thinning treatment. Seedlings from these 20 populations were planted in 1996 in a common garden experiment (eastern Spain) under uniform climatic and soil conditions. In October 2019, a 50% thinning treatment was carried out and soil samples were collected immediately before and one year after thinning.

Results Before thinning, no significant differences in soil fungal composition were observed between ecotypes. However, saprotrophic richness increased significantly in three ecotypes and saprotrophic diversity in one ecotype one year after thinning. Conversely, the ectomycorrhizal fungal community diversity and composition of the five ecotypes showed non-significant changes following thinning.

Conclusion Our results suggest that genetic differentiation in the host tree plays a minor role in shaping the ectomycorrhizal and saprotrophic communities of Mediterranean Aleppo pine forests. Furthermore, the contrasting response of the ectomycorrhizal and saprotrophic communities to thinning treatment highlights the resilience of ectomycorrhizal communities to short-term disturbances such as thinning, while emphasizing the ability of the saprotrophic communities to exploit newly available resources.

Keywords Ecotype · Ectomycorrhizal fungi · Forest management · Mediterranean pine · Population differentiation · Saprotroph

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Abbreviations

ECM	Ectomycorrhizal
Hell	Hellinger
PA	Presence–absence
OTU	Operational taxonomic unit
PMAV	Permutation multivariate analysis of variance

Introduction

In Mediterranean areas, trees and their associated fungal communities have co-evolved for millennia coping with environmental constraints such as drought and wildfires (Martin and van der Heijden 2024). In these environments, soil ectomycorrhizal (ECM) fungi are crucial for improving soil nutrient uptake by plants (Smith and Read 2008). Furthermore, soil saprotrophic fungi decompose litter and dead wood, facilitating the release of nutrients into the soil (Baldrian et al. 2011). Soil fungal communities can differ in their abundance, taxonomic composition and diversity not only between host tree species (Likulunga et al. 2021), but also within the same tree species associated with intraspecific differentiation (Johnson et al. 2012; Whitham et al. 2012). Multiple studies have highlighted the role of intraspecific genotypic variation of the host trees in modulating the ECM soil community composition. For example, Gehring et al. (2017) observed compositional differences in the ectomycorrhizal fungal communities between drought-tolerant and drought-intolerant genotypes of *Pinus edulis* Engelm. growing mixed in a stand in northern Arizona. Similarly, significant differences in the soil ectomycorrhizal community composition were found between genotypes of *Pinus pinaster* Ait. (Spain) (Pérez-Izquierdo et al. 2017, 2019) and *Pinus massoniana* Lamb. (China) (Lin et al. 2024). Conversely, there is a paucity of literature focusing on differences in soil saprotrophic communities as mediated by intraspecific variability of the tree host (Korkama et al. 2007). Nevertheless, saprotrophic fungi show a wide functional diversity (Boddy 1999; Boddy and Hiscox 2016) and a high substrate specialization (Algora Gallardo et al. 2021), suggesting that they may vary according to the intraspecific genetic background of the tree host. Against this background, a better understanding of the role of host tree genetics in determining ECM and saprotrophic soil fungal communities is needed. This is particularly important in Mediterranean forests, where tree species show diverse morphological and physiological adaptations to cope with prolonged drought at the intraspecific level (Nardini and Pitt 1999; Voltas et al. 2015).

The circum-Mediterranean conifer Aleppo pine (*Pinus halepensis* Mill.) is a species native to warm, arid environments of great ecological and socio-economic importance (Jaouadi et al. 2019). It has a wide ecological range, with populations ranging from xeric (dry) to mesic (sub-humid) areas. Drought stress has been crucial in driving the local adaptation of Aleppo pine populations (Tapias et al. 2004). Numerous studies, performed in common-garden tests or under controlled conditions, revealed genetic differentiation among populations in traits such as reproduction (Climent et al. 2008), phenology (Klein et al. 2013), seasonal water uptake dynamics (Voltas et al. 2008), water use efficiency and transpiration (Voltas et al. 2015; Santini et al. 2019a), aerial growth (Schiller and Atzmon 2009; Voltas et al. 2018), crown architecture (Santini et al. 2019b), and root morphology (Lombardi et al. 2021, 2022a). Importantly, a climate-based grouping of ecotypes closely matched the underlying genetic structure of the species (Serra-Varela et al. 2017; Patsiou et al. 2020). Thus, ecotypic variation was found to effectively summarize intraspecific functional differences within this species. In particular, Aleppo pine ecotypes show a large differentiation in tree height, mainly related to rainfall variability (Patsiou et al. 2020), in needle unfolding and senescence (Lombardi et al. 2023), and also in root system architecture (depth, diameter, and number of roots) (Lombardi et al. 2021). For instance, Patsiou et al. (2020) found that sub-humid ecotypes were growing up to 13% more in height than semi-arid ecotypes. Furthermore, Lombardi et al., (2021) found that coarse root depth mean values varied between 21 and 30 cm among ecotypes, with maximum depth reached at the thermal midpoint of the species distribution (ecotypes coming from sub-humid areas). Recent studies have shown that intraspecific variation in the roots of *Pinus massoniana* significantly influences the fungal communities in the rhizosphere (Lin et al. 2024). In this regard, the wide range of morphological

and physiological adaptations of *Pinus halepensis* ecotypes (Voltas et al. 2018) may induce differences in their associated ECM and saprotrophic soil fungal communities.

In Mediterranean forests, the intensification of fire events and prolonged droughts (Adeyeri et al. 2024) calls for the development of forest management strategies that enhance resistance and resilience to multiple stressors (Resco de Dios et al. 2007; del Campo et al. 2022). One such strategy is forest thinning, which, by removing part of the stand's timber volume, alters the competitive environment and redistributes access to resources (light, nutrients, and water) among the remaining trees (Aldea et al. 2017; Moreau et al. 2022). However, there is conflicting and limited information on the effects of thinning on soil fungal assemblages (Zhou et al. 2020b; Nuryadi 2024), suggesting that both the host tree species and the environmental conditions are playing an important role. For example, Castaño et al. (2018) found no short-term thinning effects on interannual changes in soil fungal assemblages and diversity in Mediterranean *Pinus pinaster* stands. Conversely, Caihong et al. (2023) observed significant short-term thinning effects in *Pinus koraiensis* Siebold & Zucc. plantations in China, with thinned plots showing lower abundance but higher fungal diversity compared to control plots. In any case, previous studies on the effects of thinning on soil fungal communities have been conducted, for multiple species, at the species level (e.g., *Larix* spp. [Zhou et al. (2020a, b)], *Pinus* spp. [Castaño et al. (2018)], and *Picea* spp. [Caihong et al. (2023)]), whereas little is known about the short-term effects of thinning on the soil fungal communities at the tree intraspecific level.

We used a common garden experiment of Aleppo pine (*P. halepensis* Mill.) to investigate whether the intraspecific genetic diversity of this tree species mediates short-term thinning effects on soil fungal communities. We used 20 range-wide populations with contrasting morpho-physiological characteristics at the adult stage (Patsiou et al. 2020; Santini et al. 2020; Lombardi et al. 2021), which were assigned to five ecotypes following Patsiou et al. (2020). We assessed differences in soil fungal composition and diversity both immediately before and one year after a 50% systematic thinning, using DNA-based characterization of the soil fungal community. We aimed to test two main hypotheses:

1. Differences in ectomycorrhizal and saprotrophic soil fungal community composition, richness, and diversity are associated with ecotypic differentiation in Aleppo pine.
2. The five ecotypes might respond differently to thinning, leading to differential changes in the fungal community composition, richness, and diversity compared to pre-thinning conditions.

Materials and methods

Study site

The study was conducted in a common garden (i.e. genetic trial) established in 1997 in the municipality of Altura (39°49′29″N, 00°34′22″W, 640 m a.s.l; province of Castellón, eastern Spain) (Fig. 1a). The site is representative of the average climatic conditions of the Aleppo pine distribution range, with a mean annual temperature of 13.8 °C and mean annual precipitation of 468 mm (Patsiou et al. 2020; Lombardi et al. 2022b). In February 2016, the soil properties of the common garden were determined by collecting a soil sample from each block and then pulling them to obtain a unique sample representative of the soil of the common garden. In general, the soil at the study site is a calcareous cambisol with a loamy texture (44.2% sand, 24.3% clay, and 31.5% silt) and a maximum depth of about 40 cm followed by a petrocalcic horizon with vertical fractures (visual inspection) (Lombardi et al. 2021). More information about the soil physico-chemical properties of the study area can be found in Table 1.

Common garden and thinning experiment

In 1995, seeds from 56 populations of Aleppo pine, covering most of the natural range of the species in the Mediterranean basin, were collected and sown in a forest nursery in Spain. The one-year-old seedlings were planted in the trial area with a separation of 2.5 m between seedlings, according to a latinized row-column design, resulting in 896 individuals of Aleppo pine belonging to the 56 populations. The ~1-ha trial was divided into four blocks (i.e., replicates) of 2500 m^2 each. For this study, we used three of the four available blocks, as some trees from the fourth block were affected by a fire in 2012. Within

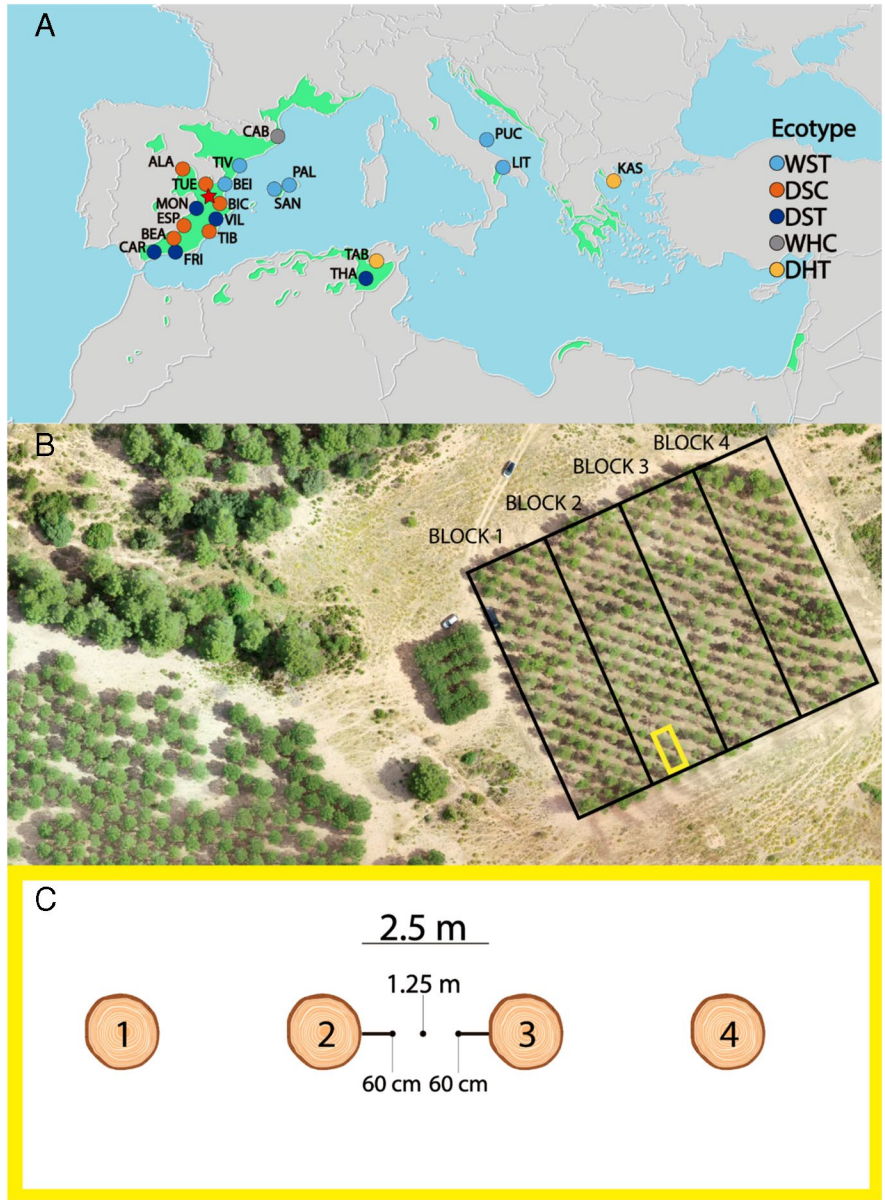


Fig. 1 **a** Geographical origin of 20 Aleppo pine populations (colored dots, according to the ecotype) growing in a common garden (red star) located in Altura, Castellón province, Spain (39°49′29″N, 00°34′22″W, 640 m a.s.l.). Population acronyms (fully described in Supplementary Data, Table S1) are shown next to each population origin (KAS = Kassandra, TAB = Tabarka, ALA = Alcantud, BEA = Benamaurel, BIC = Bicorp, ESP = Santiago de la Espada, TIB = Tibi, TUE = Tuéjar, CAR = Carratraca, FRI = Frigiliana, MON = Monovar, THA = Thala, VIL = Villajoyosa, CAB = Cabanelles, BEI = Benicàssim, LIT = Litorale Tarantino, PAL = Palma de Mallorca, PUC = Gargano Monte Pucci, SAN = Santanyí, TIV = Tivissa). The light green areas in the map represent the natural distribution of Aleppo pine according to EUFORGEN (http://www.euforgen.org/species/pinus-halepensis/). **b** Aerial picture of the trial after the thinning made in November 2019. The trial comprises four blocks (black rectangles). The yellow rectangle exemplifies an experimental unit consisting of four trees of the same population (only two remaining after the thinning). **c** Soil sampling design. We took two subsamples at approximately 60 cm from the second and the third tree within each experimental unit, while one subsample was taken halfway between the second and the third tree. The three soil cores per experimental unit (subsamples) were pooled in the field to have one sample per population in each block at each time

Table 1 Soil physico—chemical properties of the common garden in Altura, Castellón province (eastern Spain)

Analysis	Abbreviation	Unit	Results
Humidity 105 ºC		%	1.62
pH (ext. 1:2.5 H2O)			7.37
Electrical conductivity 25ºC(ext. 1:5 H2O)		dS m^{-1}	0.178
Organic matter (W&B)		%	2.79
Calcic carbonate EQUIV		%	44
Nitrogen-nitrical	N-NO3	mg kg^{-1}	< 1
Phosphorus (Olsen)	P	mg kg^{-1}	4.4
Potassium (ext. ammonium acetate)	K	mg kg^{-1}	234
Calcium (ext. ammonium acetate)	Ca	mg kg^{-1}	7464
Magnesium (ext. ammonium acetate)	Mg	mg kg^{-1}	121
Sodium (ext. ammonium acetate)	Na	mg kg^{-1}	< 15
Total sand (0.05 < D < 2 mm)		%	44.2
Coarse silt (0.02 < D < 0.05 mm)		%	9.5
Fine silt (0.002 < D < 0.02 mm)		%	22.0
Clay (D < 0.002 mm)		%	24.3
Iron (ext. EDTA)	Fe	mg kg^{-1}	104
Copper (ext. EDTA)	Cu	mg kg^{-1}	3.5
Manganese (ext. EDTA)	Mn	mg kg^{-1}	82
Zinc (ext. EDTA)	Zn	mg kg^{-1}	1.8

each block, four trees for each of the 56 populations were planted along the same row, defining a total of 56 experimental units. The last tree of an experimental unit was 2.5 m away from the first tree of the next experimental unit. The location of the experimental units was randomized within each block. A thinning treatment was carried out on October 8 th, 2019, systematically removing approximately 50% of the trees originally present in the trial (Lombardi et al. 2022b), and leaving two out of the four trees present in each experimental unit. The stump and the root system of the felled trees were left in the ground (Fig. 1b).

For this study, we selected 20 populations representative of the natural habitat of Aleppo pine. The geographical origin of the populations is described in Supplementary Data, Table S1. Following Patsiou et al., (2020), we grouped these populations into five ecotypes. Each ecotype is characterized by a particular "climate type" based on three criteria (Le Houérou 2004): first, mean summer precipitation, MSP (dry summer: MSP ≤ 60 mm; wet summer: MSP ≥ 80 mm); second, mean annual precipitation, MAP (arid–semiarid: MAP ≤ 600 mm; sub-humid: MAP > 600); and third, winter minimum temperature, TMN (cold: – 1 < TMN ≤ 1 °C; cool: 1 < TMN ≤ 3 °C; temperate: 3 < TMN ≤ 5 °C) (Patsiou et al. 2020). The ecotypes were, therefore, identified as follows: Wet-Summer/sub-Humid/Cool (WHC; one population from northeastern Spain, n = 4 trees per block), Dry-Summer/Semiarid/Temperate (DST; five populations from coastal southeastern Spain and inland Tunisia, n = 20 trees per block), Dry-Summer/sub-Humid/Temperate (DHT; two populations from Greece and Tunisia, n = 8 trees per block), Dry-Summer/Semiarid/Cold (DSC; six populations from inland Spain, n = 24 trees per block), and Wet-Summer/Semiarid/Temperate (WST; six populations from Italy, northeastern Spain and the Balearic Islands, n = 24 trees per block).

Soil sampling

Soil samples of the 20 selected populations were collected in blocks I, II, and III of the common garden on October 3rd, 2019 (right before thinning), and on October 20 th, 2020 (one year after thinning) (Fig. 1b). Within each experimental unit in each block, three soil cores (i.e., three subsamples) reaching the depth of 10 cm [which consisted of a shallow organic layer (1—2 cm) followed by mineral soil (8 −9 cm)] were taken with a hand auger (diameter = 3 cm) between the second and the third tree (Fig. 1c).

To maximize the interception of roots belonging to trees of the same ecotype (Lombardi et al. 2021), we took three subsamples per experimental unit: two subsamples at approximately 60 cm from the second and the third tree, and one subsample halfway between the second and the third tree within each experimental unit (i.e., population). The three subsamples were pooled in the field to have one sample per population in each block at each time, for a total of 120 soil samples (20 populations ×3 blocks ×2 sampling times). Once in the laboratory, the samples were lyophilized, sieved (≤ 2 mm mesh), and ground to a fine powder using a mortar and pestle. The resulting powder was stored at room temperature in a dark and dry place before the extraction of soil fungal DNA.

Soil fungal community analysis

Fungal genomic DNA was extracted from 500 mg of soil using a NucleoSpin® NSP soil kit (Macherey–Nagel, Düren, Germany). DNA concentration was measured spectrophotometrically using a nanodrop (ND-1000 Spectrophotometer, ThermoScientific), and templates were diluted to 0.5 ng μL^{-1}. The fungal Internal Transcribed Spacer 2 (ITS2) region was amplified by PCR in triplicate using the forward gITS7 primer (Ihrmark et al. 2012) [GTGARTCAT CGARTCTTTG] and the reverse ITS4 primer (White et al. 1990) [TCCTCSSCTTATTGATATGC]. Both primers were fitted with unique 8-bp tags that differed in at least three positions (Clemmensen et al. 2016) to individually identify each sample during a posteriori bioinformatic analysis. We optimized the number of PCR cycles (Castaño et al. 2020), finally using 26–29 cycles for most samples. The final concentrations in 50 µl PCR reactions were: 2.5 ng template, 200 µM of each nucleotide, 2.75 mM $MgCl_2$, primers at 200 nM, 0.025 U μL^{-1} polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA, USA) in 1× buffer. PCR cycling conditions were: 5 min at 95 °C, followed by 24–29 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. Amplified products were purified using a NucleoMag NGS Clean-up and a Macherey–Nagel size select kit, and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). We had only 80 different tags available therefore, some of the tags were used twice. To be able to differentiate the samples with the same tag, the 132 samples (120 samples from the plantation + 6 negative controls + 6 PCR controls) were divided into two pools (libraries). Equal amounts (ng) of DNA from each sample were pooled and purified using an EZNA Cycle Pure kit (Omega Bio-Tek, Norcross, GA, USA) to obtain a total of 1 µg of DNA for each library. The two libraries were sequenced on Illumina MiSeq (2 ×300 cycles) at the Centre for Genomic Regulation (CRG) Barcelona, Spain.

Quality filtering and bioinformatics analysis

MiSeq Illumina reads were provided as demultiplexed (i.e., samples were split into individual files) paired-end (i.e., forward and reverse) FASTQ files. The demultiplexed sequences (FASTQ files) were then analyzed following step by step the pipeline in PipeCraft 2 (v 1.0) (Anslan et al. 2017); pipecraft2-manual.rtfd.io/. The sequences were reoriented, and the primer strings were removed from sequences with *cutadapt* (Martin 2011). Consequently, the sequences were filtered, the paired-end reads were merged (i.e., matching forward with reverse reads) and the chimera were filtered using the VSEARCH algorithm. After quality control, one sample contained no reads and was therefore excluded. The ITS2 fungal sequences were then extracted with ITSx (Bengtsson-Palme et al. 2013). The Operational Taxonomic Units (OTUs) were then clustered using the VSEARCH algorithm (similarity threshold = 0.97) and subsequently curated using LULU (Frøslev et al. 2017). We checked the sequencing depth of the samples and found that on average the samples had 10,000 reads, with a minimum of 1771 and a maximum of 31,887 reads. Finally, the taxonomy was assigned using the latest UNITE fungal dataset (https://doi.plutof.ut.ee/doi/https://doi.org/10.15156/BIO/2483925). The FungalTraits dataset (Põlme et al. 2020) was used to assign ecological information (primary lifestyle) to OTUs. The OTU abundance table contained 3608 OTUs in 119 samples.

Statistical analysis

Statistical analyses were performed in the R software environment 4.3.3 (R Core Team 2023). To account for taxa with low number of reads (Legendre and Gallagher 2001), the soil fungal community matrix was Hellinger transformed (Hell) using the *decostand*

function from the package VEGAN v. 2.6.4 (Oksanen 2015). The package TIDYVERSE v. 2.0.0 (Wickham et al. 2019), LEMON v. 0.4.7 (McKinnon Edwards 2023) and RESHAPE2 v. 1.4.4 (Wickham 2007) were used for plot editing, and data reshaping.

The five climate-derived ecotypes of Aleppo pine, which exhibit large differences in tree height and root system architecture (Patsiou et al. 2020; Lombardi et al. 2021), helped to delineate broad patterns of potential differentiation in soil fungal communities. We analyzed the saprotrophic and ECM soil fungal community composition for each ecotype just before thinning (October 2019). We tested the ecotype effect on fungal composition using a permutation multivariate analysis of variance, PMAV, function *adonis2* from the package VEGAN v. 2.6.4 (Oksanen 2015), constrained by block (to account for each provenance being repeated in three blocks) and position within the block (to account for possible edge effects, such as differences in light affecting the microclimate and thus soil fungal communities). To visualize fungal compositional differences between ecotypes, we used non-metric multidimensional scaling (NMDS, Bray–Curtis distance), with standard deviation (SD) ellipses for each ecotype. Furthermore, we performed an indicator species analysis using the function *multipatt* from the package INDICSPECIES v. 1.7.14 (De Cáceres and Legendre 2009) and we plotted bar plots showing the relative abundance at the genus level for the ECM and saprotrophic communities of each ecotype. To increase clarity, the less abundant genera were grouped in the bar plots. To test for significant differences in the relative abundance of the eight most abundant genera between ecotypes (i.e., DHT, DSC, DST, WHC, WST) and thinning (i.e., October 2019, and October 2020), we ran a linear mixed model using the function *lme* from the package NLME v 3.1.162 (Pinheiro et al. 2023) with relative abundance of a specific genus as the response variable, ecotype or thinning as fixed factors, and block as a random factor.

In addition, we calculated the species richness and the Shannon diversity of the soil fungal community to compare these two diversity indices for the factors (i) ecotype, and (ii) thinning for each ecotype. Species richness is an alpha diversity index, and it corresponds to the number of different fungal species in each sample. Species diversity is a metric to define the diversity of the fungal community, and it has been computed using the Shannon–Wiener diversity index. Firstly, to test for significant differences between ecotypes (i.e., DHT, DSC, DST, WHC, WST) and thinning (i.e., October 2019, and October 2020), we ran a linear mixed model using the function *lme* from the package NLME v 3.1.162 (Pinheiro et al. 2023) with richness or diversity as the response variable, ecotype or thinning as fixed factors, and block as a random factor. We analyzed separately the saprotrophic and ECM fungal community. We are aware that the replication per ecotype is slightly unbalanced as ecotype DSC and WST are represented by 6 populations, ecotype DST by 5 populations, ecotype DHT by 2 populations and ecotype WHC by one population, and for this reason the results have been interpreted with caution.

Following the same methodology used to analyze the differences between ecotypes before thinning as described above (Sect. 2.6.1), we analyzed the saprotrophic and ECM soil fungal community composition, richness and diversity of the five ecotypes one year after thinning (October 2020). Furthermore, we tested the thinning effect (before vs. one year after) on fungal composition using a permutation multivariate analysis of variance, PMAV, function *adonis2* from the package VEGAN v. 2.6.4 (Oksanen 2015), constrained by block (to account for each provenance being repeated in three blocks) and position within the block (to account for possible edge effects). Finally, we used the function *envfit* from the package VEGAN v. 2.6.4 (Oksanen 2015), to calculate the significance of the "time" vector.

Results

The overall fungal community comprised 2383 OTUs in October 2019 (i.e., immediately before thinning) and 2748 OTUs in October 2020 (i.e., one year after thinning). 49% of the OTUs were identified to the genus level. Saprotrophs were the most abundant (i.e., number of reads) fungal guild in the two sampling times (October 2019, 27%; October 2020, 28%), followed by mycorrhizal species (October 2019, 6%; October 2020, 6%), plant pathogens (October 2019, 6%; October 2020, 6%), and parasites (October 2019, 3%; October 2020, 3.6%).

Soil fungal community composition, richness, and diversity before thinning

Before thinning, no significant differences were found between ecotypes, both in the ECM (PA: $p = 0.835$; Hell: $p = 0.772$) and the saprotrophic (PA: $p = 0.963$; Hell: $p = 0.552$) fungal communities (Fig. 2, Table S2).

We identified 37 genera in the ECM community, with eight genera (i.e., *Amphinema, Geopora, Helvellosebacina, Inocybe, Suillus, Thelephora, Tomentella* and *Tuber)* representing 99% of the reads. For simplicity, the less abundant ECM genera (i.e. with less than 890 reads) have been grouped in Fig. 3 and defined as 'others'. Before thinning, we found no significant differences in the relative abundance of the 8 most abundant genera among the five ecotypes ($p > 0.05$). Of the most abundant ECM genera, *Inocybe* was the most abundant, with a relative abundance of more than 25% in all ecotypes, followed by *Tuber* and *Geopora* with relative abundances between 10 and 20% in all ecotypes and *Tomentella* and *Amphinema* with relative abundances of 10–15% in all ecotypes (Fig. 3). At the species level, only four out of 137 ECM species (2%) were significantly correlated with a particular ecotype in October 2019. Interestingly, the OTU *Inocybe sp.* was only found in samples belonging to the ecotype WHC (Table S3).

For the saprotrophic community, we identified 342 genera, with eight genera (i.e., *Cadophora, Chalara,*

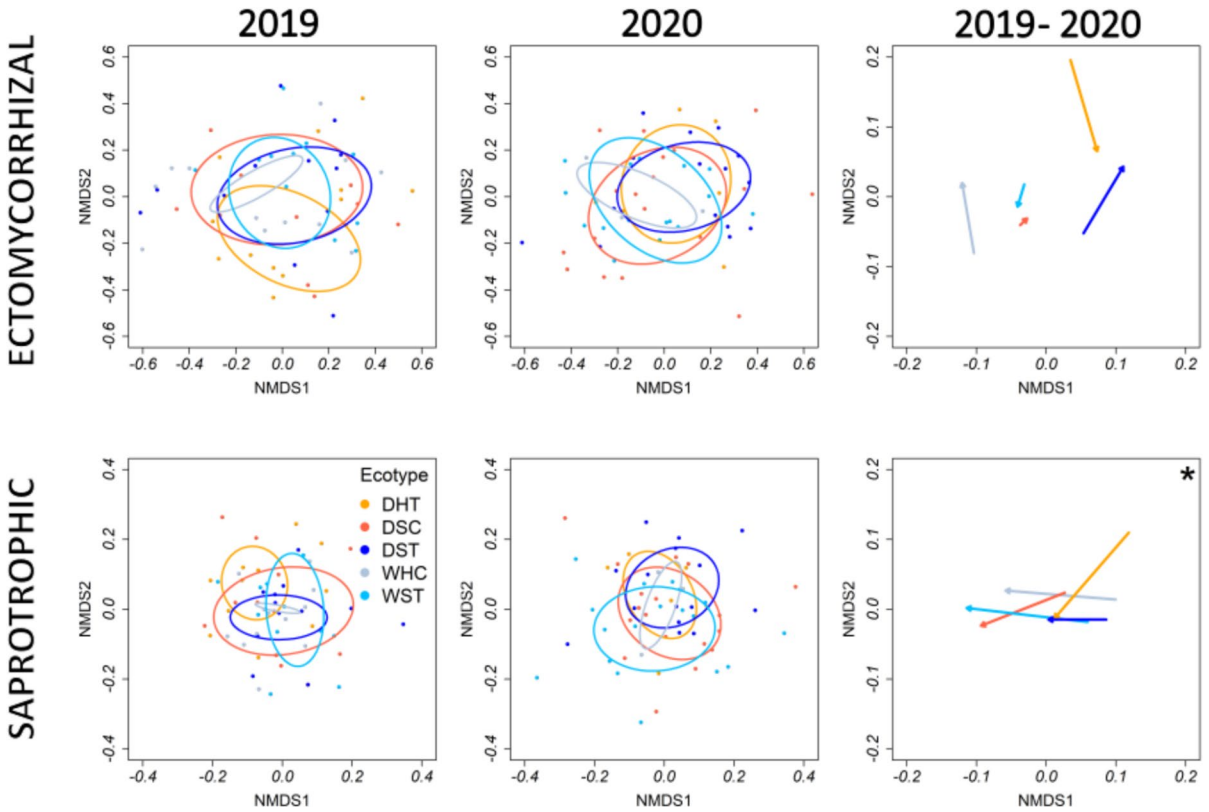


Fig. 2 Non-metric multidimensional scaling (NMDS) of the first two axes of the ectomycorrhizal (ECM, top figures) and saprotrophic (bottom figures) soil fungal community composition data of the five ecotypes in the common garden [i.e., Dry-Summer/sub-Humid/Temperate, DHT (yellow), Dry-Summer/Semiarid/Cold, DSC (orange), Dry-Summer/Semiarid/Temperate, DST (blue), Wet-Summer/sub-Humid/Cool, WHC (light gray), Wet-Summer/Semiarid/Temperate, WST (light blue)] from October 2019 (just before thinning), October 2020 (one year after thinning) and considering the two time points. Ellipses indicate the standard deviation (SD) of the community data and arrows indicate the direction of the vector from October 2019 (just before thinning) to October 2020 (one year after thinning). Where the thinning factor is significant, an asterisk has been added in the upper left corner of figure

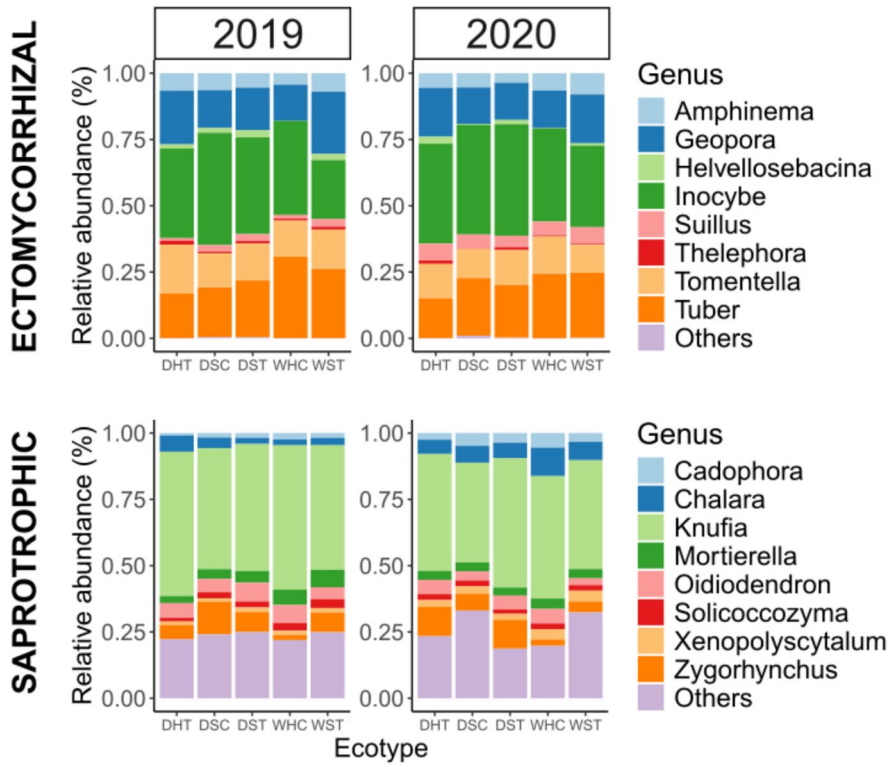


Fig. 3 Relative abundance of the eight most abundant ectomycorrhizal (ECM, top) and saprotrophic (bottom) genera of the soil fungal community of the five ecotypes in the common garden [i.e., DHT, DSC, DST, WHC, WST] in October 2019, just before thinning (2019, left panels), and October 2020, one year after thinning (2020, left panels). As the eight most abundant genera were the same in 2019 and 2020, the same colors have been used before and after thinning. The eight most abundant ECM genera had a threshold of 890 reads and the eight most abundant SAP genera had a threshold of 7400 reads

Knufia, Mortierella, Oidiodendron, Solicoccozyma, Xenopolyscytalum, and *Zygorhynchus*) representing 75% of the reads. For simplicity, the less abundant saprotrophic genera (i.e., with less than 7400 reads) have been grouped in Fig. 3 and defined as 'others'. Similar to the ECM genera, we found no significant differences in the relative abundance of the eight most abundant saprotrophic genera among the five ecotypes before thinning ($p > 0.05$). Of the most abundant saprotrophic genera, *Knufia* had a relative abundance of about 50% across all ecotypes. The genera *Zygorhynchus, Oidiodendron, and Mortierella* were also common across the ecotypes (5–15% in relative abundance). At the species level, only 16 out of 642 saprotrophic species (2%) were significantly correlated with a specific ecotype in October 2019. Four saprotrophic species were associated with the ecotype DHC, and 12 saprotrophic species were associated with the ecotype WHC. Among them, *Chaetomium spp., Dactylella zhongdianensis,* Zhang & Zhang and *Penicillium restrictum* Gilman & Abbott were found in the three blocks of the common garden, while *Oidiodendron spp.* was only found in samples belonging to the ecotype WHC. Furthermore, *Pyrenochaeta spp.* was only found in samples belonging to the ecotype DHT.

Finally, before thinning, there were no significant differences ($p > 0.05$) between ecotypes in the richness and diversity of the ECM (Fig. S1, Table S4) and saprotrophic fungal communities (Fig. 4, Table S4).

Short-term effect of thinning on the fungal community between ecotypes

The overall saprotrophic community composition (considering the five ecotypes together) showed significant differences one year after thinning (PA: $p = 0.001$; Hell: $p = 0.001$), with the thinning effect being significant and explaining 22% of the variance (envfit, $R^2 = 0.222$, $p = 0.001$) (Fig. 2). Conversely, the overall composition of the ectomycorrhizal community showed only a marginal thinning effect in the number of reads (Hell: $p = 0.069$), but not in the number of species (PA: $p = 0.560$), and the time vector was not significant (envfit: $R^2 = 0.001$, $p = 0.931$). Furthermore, we found no ecotype by thinning interaction in both the ECM and the saprotrophic soil fungal community (Table S2 c).

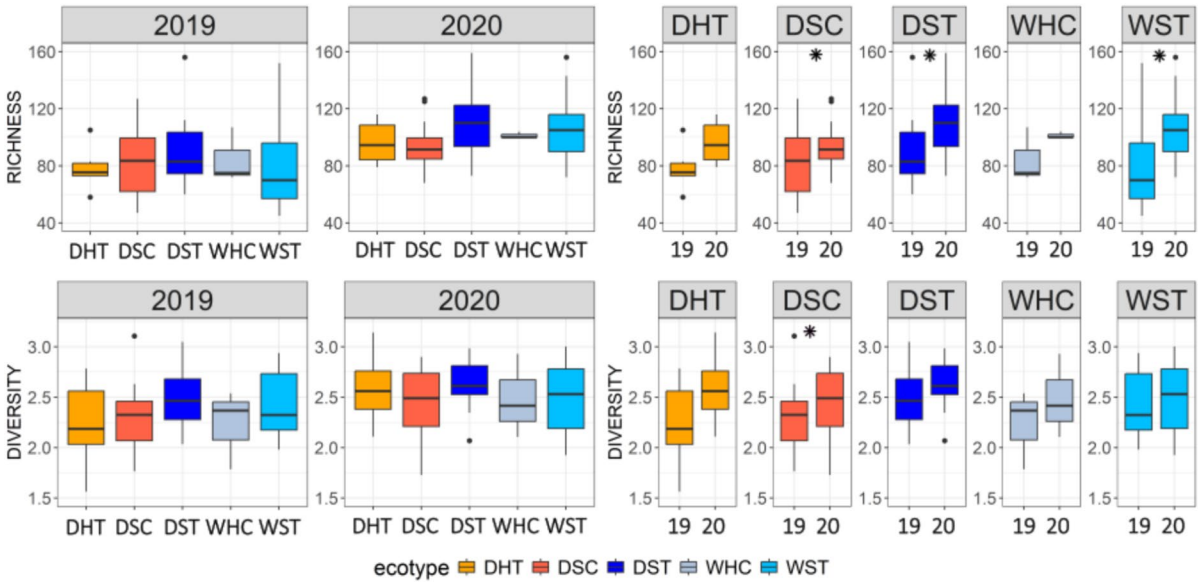


Fig. 4 Richness (total number of species) and diversity (Shannon diversity index) of saprotrophic fungal communities comparing (left panels) the five ecotypes in the common garden [i.e., DHT (yellow), DSC (orange), DST (blue), WHC (light gray), WST (light blue)] in October 2019, just before thinning (2019) and October 2020, one year after thinning (2020), (right panels) comparing each ecotype in the two time periods (i.e., 2019 and 2020). Significant differences (ANOVA, $p < 0.05$) are indicated by an asterisk at the top of the figure

We found significant differences in the number of reads between ecotypes in both the ECM (Hell: $p = 0.021$) and saprotrophic (Hell: $p = 0.004$) communities (Table S2 c). Nevertheless, when considering a single time point (i.e., either October 2019 or October 2020), no significant differences between ecotypes were found in the composition of both the ECM (PA: $p = 0.643$; Hell: $p = 0.767$) and saprotrophic (PA: $p = 0.559$; Hell: $p = 0.389$) soil fungal communities (Fig. 2, Table S2 a and b).

The relative abundance of the eight most abundant ECM genera after thinning was similar to the relative abundance before thinning. Although after thinning, no significant differences was found in the relative abundance of the 8 most abundant ECM genera between the five ecotypes, we detected a significant increase in the relative abundance of *Suillus* ($p < 0.05$) and a significant decrease in the relative abundance of *Tomentella* across all ecotypes ($p < 0.05$). At the species level, only three out of 147 ECM species (2%) were significantly associated with a specific ecotype in October 2020. Interestingly, *Trichophaea hybrida* T. Schumach was significantly correlated with the ecotype DHT both before and after thinning.

The relative abundance of the eight most abundant saprotrophic genera after thinning was similar to the relative abundance before thinning. Although after thinning, no significant difference was found in the relative abundance of the 8 most abundant saprotrophic genera between the five ecotypes, we detected a significant increase ($p < 0.05$) in the relative abundance of *Cadosphora*, *Chalara*, and *Xenopolyscytalum*, and a significant decrease ($p < 0.05$) in the relative abundance of *Mortierella* and *Oidiodendron* across all ecotypes. Furthermore, compared to pre-thinning conditions, there was an increase in the relative abundance of the less abundant genera (classified as "Others" in Fig. 3) in the DSC and WST ecotypes. At the species level, only 24 out of 773 saprotrophic species (3%) were significantly correlated with a specific ecotype in October 2020. In particular, 19 saprotrophic species were associated with the ecotype WHC after thinning. Among them, *Myxotrichum chartarum* Kunze was found in the three blocks of the common garden, while *Archaeorhizomyces sp.19, Bovista tomentosa* Vittad., *Calcarisporiella, Chalara holubovae* Koukol, *Corticium sp.3, Merulicium fusisporum* (Romell) J.Erikss. & Ryvarden,

Paraconiothyrium sp., and *Phaeothecoidea sp.* were found only in samples belonging to this ecotype. *Arrhenia elegans* (Pers.) Redhead, Lutzoni, Moncalvo & Vilgalys was significantly correlated with the ecotype WHC, both before and after thinning. Furthermore, *Capronia sp.* was only found in samples belonging to ecotype DST.

Furthermore, one year after thinning, there were no significant differences in richness and diversity of the ECM (Fig. S1, Table S4) and saprotrophic (Fig. 4, Table S4) fungal communities between ecotypes ($p > 0.05$). Interestingly, the ECM fungal community did not show significant differences in richness and diversity before and one year after thinning (Fig. S1, Table S4). Conversely, the saprotrophic fungal richness of ecotypes DSC, DST, and WST and the diversity of the ecotype DSC showed a significant increase compared to pre-thinning conditions (Fig. 4, Table S4 b).

Discussion

Our research showed that before the thinning treatment, the 20 populations of *P. halepensis,* grouped into five ecotypes, had no significant differences in their associated soil fungal communities. Similarly, one year after a 50% thinning treatment, there were no significant intraspecific differences in the ectomycorrhizal and saprotrophic soil fungal community of the five ecotypes. However, we found significant thinning effects on the saprotrophic community.

Soil fungal community composition, richness, and diversity before thinning

Contrary to our expectations, the ECM and the saprotrophic soil fungal community did not show significant differences mediated by ecotypic differentiation prior to thinning (i.e., October 2019). Previous studies have shown that, in semi-arid ecosystems such as Mediterranean forests, ectomycorrhizal (ECM) fungi can alleviate drought stress for host trees by facilitating nutrient acquisition under water-limited conditions (Castaño et al. 2017). It has also been shown that, even at the intraspecific level, the presence of a particular soil ECM community can determine a significant difference in the aerial growth of the plant. For example, Gehring et al. (2017) observed that under drought conditions, drought tolerant seedlings of *Pinus edulis* Engelm. had 25% higher aerial growth than drought sensitive seedlings of the same species, highlighting the role of the host tree genetics in defining the associated ECM community. At our study site, drought-tolerant and drought-sensitive populations of *P halepensis* Mill. have been growing side by side for more than 25 years. The climatic conditions of our study site are similar to those of drought-tolerant ecotypes originating from western semi-arid Mediterranean areas (e.g., ecotypes DSC and DST), suggesting that the local conditions should favor the western ecotypes. However, recent studies conducted in this common garden have shown that drought-sensitive ecotypes originating from eastern sub-humid areas (e.g., ecotype DHT) out-compete the local semi-arid ecotypes (Lombardi et al. 2021). In particular, the eastern ecotypes reach significantly higher tree heights (Patsiou et al. 2020) and root diameters and depths (Lombardi et al. 2021) compared to the western ecotypes. We therefore hypothesized that this performance could be related to differences in the ECM soil fungal community associated with a specific host tree.

Although the differences between ecotypes were not significant at the community level, we found that one ECM species was correlated with the DHT ecotype (*Trichophaea hybrida*), while three ECM species were strictly associated with the WHC ecotype (*Inocybe sp., Tuber malenconii,* Don, Riou, & Chev and *Tuber melanosporum* Vittad.). We also hypothesized that we would find differences in the saprotrophic soil fungal community. Although we did not find significant intraspecific differences at the community level, we did find that 2% of the species were strictly associated with a particular ecotype. In particular, four species were associated with the DHT ecotype and 12 species with the WHC ecotype. This suggests that part of the saprotrophic fungal community had a particular affinity for some *P. halepensis* ecotypes. However, in our common garden, the crowns of adult trees may overlap, so that the needles falling to the ground create a mixed litter layer, which is likely to reduce the differences in saprotrophic fungi present in the soil (i.e., below the needle litter). Interestingly, Korkama et al., (2007) studied eight *Picea abies* clones with a twofold difference in height and found that their needles differed in chemical composition, but the community structure of the fungal decomposers did not show significant differences.

In any case, possible caveats related to the identification of specific soil fungal communities presented here are related to the proximity of the adult trees in the common garden, and to the sampling method used to define the fungal community composition. The Aleppo pine trees present in the common garden have been growing together (2.5 m apart from each other) for more than 25 years. During this time, their root systems have spread into the soil and may have intermingled. In this context, Lombardi et al. (2021) used a ground-penetrating radar to characterize intraspecific variability in the coarse roots (3 to 5 cm in diameter) of the pines locate in the common garden used in this study. They have shown that there is a high concentration of roots in the first 50–60 cm around each individual tree and a subsequent decrease, suggesting that sampling soil close to the stump increases the likelihood of finding the roots of a specific individual. Furthermore, in the current study, to minimize the risk of intercepting roots from neighboring experimental units, we took three subsamples between the second and the third tree within each experimental unit, using the first and the fourth trees as a buffer zone.

Moreover, soil is often considered a reliable proxy for characterizing root fungal communities (Taberlet et al. 2018). Nevertheless, to identify tree effects on fungal communities, it may be convenient to trace roots of each host tree and extract fungal DNA directly from rhizospheric soil, near to the root tips of the host trees, as they harbor higher concentrations of root-associated fungi (Gehring et al. 2014). In contrast, we sampled bulk soil surrounding the tree stumps and extracted the fungal DNA from the soil, which may include fungal species not directly associated with the root systems. Thus, our results may reflect broader soil fungal communities rather than just the root-associated fungal populations that would be directly influenced by the tree ecotype.

Short-term effect of thinning on fungal community between ecotypes

Thinning treatments consist in removing a fraction of trees within a stand, redistributing access to light, nutrients, and water, among the remaining trees (Sohn et al. 2016). In our experiment, the aerial biomass of 50% of the standing trees was removed, while the stumps were left on the ground.

The thinning treatment may have caused a change in the local microclimate by allowing more light to reach the soil surface (Centenaro et al. 2023). Thinning also reduced litterfall. Both the change in the microclimate and the reduction in the new plant organic matter could have affected the dynamics of litter decomposition and, consequently, the saprotrophic community. In fact, we have observed that, one year after thinning, the saprotrophic richness increased significantly for the ecotypes DSC, DST, and WST. Furthermore, the saprotrophic diversity of the ecotype DSC increased significantly. This is consistent with previous work describing increased saprotrophic macrofungal diversity (Lin et al. 2011) and fungal decomposers in the litter fraction (Lagomarsino et al. 2020) after thinning. Interestingly, Lombardi et al. (2023) found that sub-humid ecotypes (i.e., WHC, DHT) exhibited earlier needle unfolding and delayed needle senescence than semi-arid ecotypes (i.e., DSC, DST, WST). The delay in needle senescence and consequent degradation of secondary organic compounds in the needle litter may have influenced the composition of the saprotrophic fungi in the soil, causing intraspecific differences in richness and diversity (Kainulainen and Holopainen 2002).

On the other hand, we hypothesized that the decrease in photosynthates might affect the ECM fungal community after thinning. However, our results show that the overall ECM community did not change significantly, and that thinning did not induce intraspecific differences in the composition of the ECM fungal community in the common garden plantation. This suggests that the five ecotypes share a common pool of ECM species.

From a management perspective, it seems clear that the intensity of thinning used in the common garden is sufficient to maintain and, in the case of the saprotrophic community, increase the richness and diversity of the soil fungal community of the five ecotypes in the short term. However, the removal of 50% of the trees from the common garden may have reduced the fungal biomass (not considered in the current study), as shown by previous studies (Collado et al. 2020). Furthermore, the lack of intraspecific differences before thinning could be related to the fact that the common garden had a homogeneous soil fungal composition when the seedlings were planted and the differences between ecotypes could have been limited due to the reduced pool of fungal species present in this area.

Conclusion

This study provides new insights into the variability of the soil fungal community mediated by intraspecific differentiation in *Pinus halepensis* before and after thinning, considering the parallel changes in the soil saprotrophic and ectomycorrhizal fungal communities. Before and one year after thinning, the five ecotypes showed no intraspecific differences in their soil fungal community composition. However, one year after thinning, the total saprotrophic community showed significant changes, with the three semiarid ecotypes showing a significantly higher richness (and one of the three semiarid ecotypes also showing an increase in saprotrophic diversity). Conversely, the thinning had no significant effect on the ECM community. Our study sheds light on the dynamics of soil fungi in Mediterranean forests in relation to tree ecology and forest ecosystem functioning. In particular, it highlights the resilience of ectomycorrhizal fungal communities to short-term disturbances such as thinning, while emphasizing the ability of the saprotrophic communities to exploit newly available resources, regardless of the intraspecific genetic background of the host tree. Nevertheless, to better elucidate the intraspecific variation in soil fungal communities associated with *Pinus halepensis* Mill. or other host trees, future studies could include rhizospheric soil samples and trace the roots back to the tree. In addition, the diversity and richness of ectomycorrhizal and/or saprotrophic fungi could be modelled in relation to tree fitness parameters (e.g., diameter, needle nutrient content).

Author contribution Giada Centenaro: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review and editing. Sergio de-Miguel: Conceptualization, Funding acquisition, Investigation, Supervision, Project administration, Writing – review and editing. Jordi Voltas: Conceptualization, Funding acquisition, Investigation, Supervision, Project administration, Writing – review and editing. José Antonio Bonet: Conceptualization, Funding acquisition, Investigation, Supervision, Project administration, Writing – review and editing. Svetlana Dashevskaya: Methodology, Writing – review and editing. Josu G. Alday: Conceptualization, Funding acquisition, Investigation, Supervision, Project administration, Methodology, Writing – review and editing.

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

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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