Lack of thinning effects over inter-annual changes in soil fungal community and diversity in a Mediterranean pine forest

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Predicted changes in global climate might negatively affect the soil microbiome and associated ecosystem processes in Mediterranean forests. Forest treatments, such as forest thinning, have been suggested to mitigate climate change impacts on vegetation by reducing competition between trees, thus increasing water availability. Studies addressing the combined effects of climate and forest thinning on belowground fungal communities are still scarce, being fundamental to elaborate adaptive strategies to global warming.

The aim of this study was to evaluate the short-term tree density reduction effects on soil fungal communities and their response to inter-annual changes in weather conditions. The temporal dynamics of soil fungal communities in relation to these two drivers (i.e., forest management and weather conditions) were studied from 2009 until 2014 in a set of 12 pairs of thinned and un-thinned plots dominated by *Pinus pinaster* Ait. Thinning (from 30% up to 70% reduction in stand basal area) was conducted in 2009 and soil fungal community composition was studied during 4 years. Here, we used autumn precipitation and temperature to describe the impact of inter-annual weather changes. We used Pacific Biosciences sequencing of fungal ITS2 amplicons to study fungal communities in soil samples. Forest thinning did not significantly affect fungal community composition nor fungal species richness and diversity, indicating that the soil fungal community in the short-term is resistant to forest thinning regardless of its intensity. However, fungal species composition changed progressively across years, both at the species level and with regards to functional guilds. These changes in community composition were partly driven by inter-annual variation in precipitation and temperature, with free-living fungi increasing in abundance under wetter conditions, and symbiotic fungi being more prominent under drier and colder conditions. The
results indicate that mycorrhizal communities in Mediterranean forest ecosystems can resist forest thinning, if enough trees and functional roots from thinned trees are retained.

Keywords: forest management, mycorrhizal, climate, drought, saprotrophs, fungal diversity

1. Introduction

Soil fungi represent an important part of the soil microbial community, and are essential drivers of many ecosystem processes, such as soil organic matter (SOM) decomposition and nutrient release as well as plant nutrient uptake and production. Mycorrhizal fungi are one of the most important functional groups of the soil microbiome, playing an important role in tree nutrition and water acquisition (Smith and Read, 2008). The extramatrical mycelia (EMM) of these fungi explore the soil surrounding the host tree, foraging for nutrients and forming mycorrhizae with adjacent tree hosts (Cairney, 2012). In drier ecosystems, such as Mediterranean forests, mycorrhizal fungi contribute to plant water acquisition, by providing plant roots access to less accessible water and by improving soil structure, enhancing soil water retention (Allen, 2007; Querejeta, 2017). Besides the important role of mycorrhizal fungal species, other functional guilds, such as saprotrophs, also play a paramount role in litter degradation (Baldrian et al., 2011), which may be hampered during the dry and hot summer conditions of Mediterranean forest soils. Thus, fungal community changes in these ecosystems will have important consequences for nutrient cycling and water acquisition by plants and therefore impact plant communities (Sardans and Peñuelas, 2013).

Global change is one of the most important threat for many Mediterranean ecosystems. In the Mediterranean basin, temperature has been forecasted to rise between 1.4°C and
5.1°C by 2055 (Nogués Bravo et al., 2008), and total annual precipitation projections show a tendency towards less precipitation, with more extreme rainfall events (García-Ruiz et al., 2011) and reduced soil moisture (Dai, 2013). Indeed, ecosystem alterations, local extinctions and phenological changes in these ecosystems have already been associated to current climate change (Peñuelas et al., 2002). Also, predicted drought increase in Mediterranean forests will likely reduce plant growth and aboveground biomass (Sardans and Peñuelas, 2013), probably with cascading effects belowground (Cairney, 2012; Alday et al., 2017a). Thus, changes in climate may alter the composition of soil fungal communities (Fernandez et al., 2016; Solly et al., 2017; Hartmann et al., 2017; Castaño et al., 2018) and cause alterations in ecosystem functioning with respect to plant nutrition, soil organic matter decomposition and carbon storage (Averill et al., 2014; Clemmensen et al., 2015).

Climate effects on fungi may be directly driven by changes in temperature and moisture (Voříšková et al., 2013; Santalahti et al., 2016) or indirectly by changes in host performance (Deslippe et al., 2011; Fernandez et al., 2016; Hartmann et al., 2017), host activity (Högberg et al., 2010), soil properties or litter input (Vašutová et al., 2016). In drier ecosystems, some mycorrhizal ascomycetes may be more abundant (Smith et al., 2007; Gordon and Gehring, 2011). Precipitation and temperature strongly influence positively fruiting body emergence and production (Hernández-Rodriguez et al., 2015; Alday et al., 2017b). Fire severity and thinning also affect fungal fruit body production (de-Miguel et al., 2014; Salo et al., 2018) as well as community composition of fruiting bodies (Mediavilla et al., 2014). Recently, we observed that intra-annual changes in soil microclimate conditions strongly affected belowground fungal functional communities (Castaño et al., 2018). In addition, Léon Sánchez et al., (2017) studied the climate change effects on the belowground fungal community in a scrubland, and they observed
negative effects of drier and warmer conditions on mycorrhizal species. However, studies focussed on belowground fungal responses to both thinning and climate changes in Mediterranean forests are still scarce. Forest thinning has been suggested as a forest management option to mitigate climate change impacts on Mediterranean forests, because its potential to increase water availability and water use efficiency of trees, thus changing soil microclimatic conditions. For example, Aldea et al. (2017) found that thinning increased radial growth of both conifer and oak species and led to increased resistance to drought and improved stand growth. Similarly, positive thinning effects have been observed on the fruiting body production of economically relevant fungal species (Shaw et al., 2003; Bonet et al., 2012), although the effects were species-dependent. Also, sustainable forest harvesting regimes have been predicted to positively influence mushroom production (de-Miguel et al., 2014). In contrast, clear-cutting and associated logging disturbances in clear-cut forests have been shown to have clear negative impact on soil mycorrhizal communities (Jones et al., 2003; Hartmann et al., 2012; Kyaschenko et al., 2017; Parladé et al., 2017). Forest management effects on mycorrhizal communities are likely to depend on whether these communities can survive in symbiosis with the remaining trees (Amaranthus and Perry, 1987; Rosenvald and Lõhmus, 2008). Tree removal may also affect belowground fungal communities via changes in environmental conditions, such as microclimate or soil biochemistry (Jones et al., 2003; Hartmann et al., 2012). Although forest thinning may have a less dramatic impact than clear-cutting, its impact on belowground fungal communities has yet not been assessed.

In this study we analysed the inter-annual dynamics of soil fungal communities during 4 years after forest thinning in 12 experimental plots dominated by Pinus pinaster Ait, with 12 paired non-thinned plots as a reference. The plots represented a gradient of
retained stand basal area and number of trees (Bonet et al., 2012). In addition, we analysed potential correlations between autumn precipitation and temperature and the fungal community composition and structure. In recent studies, we analyzed the soil microclimate effects on fungal communities from an intra-annual perspective, with significant effects found (Castaño et al., 2018). However, here we study both the climate and thinning effects from an inter-annual perspective. We specifically hypothesized that i) light-medium thinning would not alter belowground fungal community composition or diversity. In contrast, ii) changes in fungal species composition and the relative abundance of functional guilds would be expected after more intense thinning. We further hypothesized that iii) fungal community composition would vary across years in relation to autumn precipitation and temperature, with mould species and yeasts being stimulated under wetter conditions.

2. Material and Methods

2.1 Site selection

The study was carried out at a long-term experimental setup located in the natural area of PNIN-Poblet (Northeast Spain, 41° 21’ 6.4728” latitude and 1° 2’ 25.7496” longitude), where 12 pairs of thinned and non-thinned plots were established in 2009 to test the effect of forest thinning on mushroom production (Bonet et al., 2012). The plots consist of even-aged Pinus pinaster stands (60-years-old), with isolated Quercus ilex trees sometimes forming shrubs, while the understory is dominated by Erica arborea, Arbutus unedo and Calluna vulgaris. Mean annual temperature at the study site is 11.8 °C, and mean annual rainfall is 666.5 mm, with a pronounced summer drought that usually lasts for three months (June to August). Autumn precipitation (September to November) during the study years was similar across plots (136.8±3 mm), but variable between years (136.8±86.4 mm), whereas temperature variation was slightly higher
across plots (16.06±1.13 °C) than across years (16.06±0.84 °C). Averaged autumn rainfall was: 2009 = 97.1±3.4 mm, 2012 = 245.9±22.7 mm, 2013 = 30.59±0.3 mm, 2014 = 108.9±18.6 mm. Yearly averaged autumn temperature was: 2009 = 17.1±0.9 °C, 2012 = 16.4±0.9 °C, 2013 = 15.3±1.5 °C, 2014 = 15.4±1.2 °C. Plots are similar in soil properties, but as a result of 2009 thinning, their characteristics differ considerably, with basal area ranging from 16.5 to 81.7 m² ha⁻¹ and stand density from 350 to 2,657 trees ha⁻¹. Soils are siliceous with sandy loam texture, average pH 6.7±0.3, average total N 0.21±0.06% and organic matter (OM) 5.5±2%.

2.2 Thinning experiment

Initially, 12 mushroom inventory plots of 100 m² (10m × 10 m) were established in 2008 (un-thinned plots) in an approximately 300 ha forest area (Fig. S1a). In 2009 12 additional inventory plots, scheduled for thinning (thinned plots), were established paired (with an average distance of 50 m. from controls) with the initial plots. Each thinned plot was 1600 m² in area (40m×40m) with a central 100m² sampling area, to reduce edge effects. In these thinned plots, three different thinning intensities were employed (light: 20-30% thinned, medium: 30%-50% thinned, and heavy: 50-70% thinned), resulting in basal area reductions of 30% to 70% (Fig. S1b). In un-thinned plots, stand structure was more or less homogeneous, with similar tree heights and diameters within plots. In thinned plots, trees were systematically removed without the use of heavy machinery to avoid confounding effects caused by soil disturbance, using a chainsaw and removing the cut trees from the plot. The most intense thinning resulted in a remaining stand basal area of 16.5 m² ha⁻¹ and a stand density of 350 trees ha⁻¹, whereas the greatest standing basal area left was 81.7 m² (2,552 trees ha⁻¹). Further information about the thinning treatments and the stand variables before and after the
treatments is available in Bonet et al. (2012). A diagram of the experimental design is provided as Fig S1.

2.3 Soil sampling

Soil sampling was conducted in all 24 plots in November 2009, 2012, 2013 and 2014 (Fig. S1c). Since soil was not sampled in 2010 and 2011 due to funding limitations, our focus was on the immediate thinning effect (2009) and the medium term effect (2012-2014). Each year, eight soil cores (12 cm deep and 5 cm in diameter) were systematically sampled with a metallic probe in each plot (two along each side of the plot). In these samplings, needles and partially decomposed needles were excluded, since fungal community composition in the duff layer mostly consists of saprotrophic fungi (Clemmensen et al., 2013; Voříšková et al., 2013), whereas humus and mineral soil were sampled together. Soil samples were sieved using 3mm mesh, stored at 4°C during <24h, freeze-dried and pooled by plots. Each of the 96 composite soil samples (24 plots × 4 years) was ground to fine powder using mortar and pestle. The resulting fine powder was stored at -20°C before DNA extraction.

2.4 Soil fungal community analysis

Genomic fungal DNA was extracted from 500 mg of soil using the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer’s protocol, but with 900 µl of lysis buffer (SL1). The ITS2 region was PCR amplified in a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) using the primers gITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990). Both primers were fitted with unique 8-bp tags, differing in at least three positions. The number of PCR cycles was optimized for each individual sample, with most of the samples amplifying well at 21-24 cycles. Each sample was amplified in triplicates with negative extraction and PCR controls. Final concentrations in the 50 µl PCR reaction mixtures were: 25 ng template,
200 µM of each nucleotide, 2.75 mM MgCl2, primers at 200 nM, 0.025 U µL⁻¹ polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA) in 1X buffer PCR. PCR cycling conditions were as follows: 5 min at 95°C, followed by 24-30 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 before storage at 4 °C. PCR products were purified using the AMPure kit (Beckman Coulter Inc. Brea, CA, USA) and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled and purified using the EZNA Cycle Pure kit (Omega Bio-Tek). Quality control of purified amplicons was carried out using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA) and a 7500 DNA chip. Samples were sequenced at SciLifeLab NGI, Uppsala, Sweden on a PacBio RS II system.

2.5 Quality control and bioinformatic analysis
Quality control, filtering and sequence clustering were conducted with the SCATA pipeline (scata.mykopat.slu.se). Sequences < 200 bp in length were removed and remaining sequences were screened for primers (requiring 90% sequence match) and sample tags. After collapsing homopolymers to 3 bp, sequences were pair-wise compared with ‘usearch’ (Edgar, 2011). Pairwise alignments were scored using a mismatch penalty of 1, a gap open penalty of 0 and a gap extension penalty of 1. Sequences were clustered into operational taxonomic units (OTUs) based on the Species Hypothesis (SHs) concept (Koljalg et al., 2013) using single linkage clustering with a maximum distance of 1.5% to the closest neighbour required to enter clusters. Sequence data are archived at NCBI’s Sequence Read Archive under accession number PRJNA309233 (www.ncbi.nlm.nih.gov/sra).
2.6 Taxonomic and functional identification

We assigned putative taxonomical identities to the 500 most abundant SHs, representing 93% of the total, high-quality DNA sequence reads. The most abundant sequence from each SH was selected for taxonomical identification, using the massBLASTer in PlutoF against the UNITE (Abarenkov et al., 2010) and INSD databases. Taxonomic identities were assigned to SHs with closed matches (> 98.5% similar) to database references, or based on well supported monophyletic neighbour-joining clades including database references. Functional roles of SHs were assigned as follows: a) ectomycorrhizal b) root-associated ascomycetes, c) moulds, d) yeasts, e) black yeasts, f) other saprotrophs or litter-decay fungi, g) soil saprotrophs, h) pathogens, and i) moss-associated fungi. Classification was confirmed using FUNGuild (Nguyen et al., 2016). Ectomycorrhizal SHs were assigned to exploration types based on DEEMY (www.deemy.de) and Agerer, (2001, 2006). Taxonomical, functional and exploration types assignments are shown in Table S1.

2.7 Climate data

We obtained weather variables (precipitation and temperature) from 2009, 2012, 2013, 2014 (September, October, November) for each of the 24 plots, following the DAYMET methodology (Thornton et al., 2000), as implemented in the R package ‘meteoland’ (De Cáceres et al., 2017). In short, daily precipitation and temperature were estimated for each plot by averaging the values of several Catalan and Spanish meteorological stations, applying weighting factors that depended on the geographic proximity to the target plot and correcting for differences in elevation between the station and the target plot. We used the average precipitation and temperature for September-October. Although samples were collected in late November, we did not
consider the precipitation of November because rainfall was concentrated to the third and fourth weeks of the month, when sampling was already conducted.

2.8 Data analysis

The fungal community data set was subjected to multivariate analyses using CANOCO version 5.0 (Biometris Plant Research International, Wageningen, The Netherlands) and the “nlme” package for linear mixed-effects models (LME; Pinheiro et al., 2016) in R (version 3.0.2; R Development Core Team 2013). Species data were square-root transformed to account for taxa with many zeros and low count numbers, and only SHs with more than 5 occurrences were included.

2.8.1 The effect of forest thinning on fungal community composition

Principal Response Curves (PRC) were used to evaluate effects of forest thinning effect on fungal community composition. This method is similar to partial redundancy analysis, which enables identification of time-specific treatment effects (Thinning treatments × time interaction) while controlling for the overall temporal trend, using time as a co-variable (Alday and Marrs, 2014). Here, year was defined as a factor with 4 levels (2009, 2012, 2013, 2014), whereas thinning intensity (% reduction in basal area) was defined as explanatory factor with 4 levels (control: 0% thinned, light: 20-30% thinned, medium: 30%-50% thinned, and heavy: 50-70% thinned). The thinning effect was tested for significance using Monte Carlo simulations (999 permutations).

Similarly, the short-term effects of forest thinning were tested considering only data from 2009 with the fungal community composition as response variable and either the basal area or the number of trees removed or left as explanatory variables. Three independent tests with (i) the relative abundance of SHs, (ii) the relative abundances of functional guilds, and (iii) the relative abundances of exploration types within the ectomycorrhizal community as response data.
2.8.2 Inter-annual changes in fungal community composition

A graphical representation of the fungal community similarity between years was obtained by Detrended correspondence analysis (DCA). The significance of changes in fungal community composition across years was tested using Canonical correspondence analysis (CCA) with plot identity as a covariate and years randomly permuted (999 permutations) in a Monte Carlo test. Year was included both as a factor and as a quantitative variable in separate analyses. Thus, tests were carried out without permutation between spatial replicates within single years. This test was performed in three independent response datasets: (i) the relative abundances of SHs, (ii) the relative abundances of functional guilds and (iii) the relative abundances of exploration types within the ectomycorrhizal community. PRC were also used to visualize changes in community composition across years. Here, plot identity was defined as a covariate, and year was defined as a factor with 4 levels and randomly permuted (999 permutations) in a Monte Carlo test. We also tested whether yearly changes in fungal community composition were correlated with autumn precipitation and temperature, using a CCA with the same permutation scheme, but using forward selection of explanatory variables to disentangle the proportion of explained variation from each variable (precipitation and temperature). We used only autumn precipitation because previous studies using soil samples from the same site indicated fast responses of the fungal community to changes in soil moisture and temperature (Castaño et al., 2017). Changes in relative abundance of each functional guild and exploration type in response to changes in precipitation and temperature were studied using linear mixed-effects (LME) models from square-root transformed relative proportions. Here, plot was defined as random factor and autumn temperature and precipitation were defined as fixed terms.
2.8.3 Thinning and climatic effects on fungal richness and diversity

Hill’s series of diversity indices were used to compare differences in diversity values between years and between thinning intensities (Hill, 1973), considering in separate analyses the whole and the ectomycorrhizal community. Hill’s diversity consists of three numbers: N0 is species richness; N1 is the antilogarithm of Shannon’s diversity index; and N2 is the inverse of Simpson’s diversity index. We did not rarefy the fungal community due to the potential information loss. Instead, we included square-root transformed read counts as an explaining variable (Bálint et al., 2015), to account for variation in sequencing depth. LME models were used to test significant changes in Hill’s numbers between years and due to thinning. In these analyses, plot identity was defined as a random factor, whereas year identity and thinning intensity, as well as their interaction, were defined as fixed factors. The same analysis based on rarefied samples (richness values calculated after subsampling to 276 sequences per sample) yielded the same results (data not shown). Similarly, short-term effects of forest thinning were tested using LME only considering community data from 2009 with plot as a random factor and thinning intensity as a fixed factor.

3. Results

3.1 Sequencing output and general community composition

We obtained a total of 75,608 ITS2 sequences after quality control. Single linkage clustering (1.5%) resulted in 2,134 SHs after removing singletons, of which 500 (93% of the high-quality sequences) were assessed for identification at the level of species, functional guilds and exploration strategy. Overall, Basidiomycota was the most abundant phyla (54±1%), followed by Ascomycota (32±1%). The most abundant guild was ectomycorrhizal fungi, representing 65±2% of the sequences, followed by moulds (13.1±0.9%), black yeasts (4.3±0.3%), root-associated fungi (3.7±0.6%), and other

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saprotrophs, most of them classified as litter saprotrophs (3.5±0.4%). Species with short and contact exploration types represented 33.2±2% and 24.6±1%, respectively, of the sequences assigned to the ectomycorrhizal guild followed by medium fringe (22.2±2%) and long exploration types (9.7%±2%).

3.2 Fungal community responses to forest thinning

Forest thinning did not have a significant effect on fungal community composition (F=1.6, P= 0.621; Fig. S2), functional guilds (F=6.0, P=0.255) or the distribution of exploration type among mycorrhizal fungi (F=4.7, P=0.576). Similarly, no significant relationship between community composition and basal area after thinning could be demonstrated (F=1.9, P=0.584). Immediate thinning effects (i.e., in year 2009) were also not significant (F=0.9, P=0.895).

3.3 Inter-annual variation of the fungal community

Fungal species composition varied significantly across years (Fig. 1a, 1b) in a progressive manner (Fig. 1a, Fig. S3), with sampling year explaining 8.2% of the total inertia (CCA pseudo-F=2.2, P=0.001).

Relative proportions of many mould species (Penicillium, Umbelopsis, Mortierella), yeasts (Cryptococcus, Guehomyces) and potential plant pathogens (Sydowia, Phoma) were more abundant early in the study, whereas relative proportions from several ectomycorrhizal species (Inocybe, Amphinema) increased with time (Fig 1b). In general, there was a progressive increase in ectomycorrhizal species over the years, from 2009 to 2014 (Fig. 1b, 2a), but exploration type proportions among mycorrhizal species did not change across years (pseudo-F=1.1, P=0.665).
Fig. 1. Changes in soil fungal community composition across years. (a) Sample plot of a Detrended correspondence analyses (DCA) based on species level fungal community composition with different colours indicating sampling year and (b) the corresponding species plot with colours corresponding to functional groups. Pseu_pann (Pseudogymnoascus pannorum), Humic_nigr (Humicola nigra), Crypto_terr (Cryptococcus terricola), Gemini_sp (Geminibasidium_sp), Suill_bell (Suillus belliini), Oidio_sp (Oidiodendron_sp), Crypt_sp (Cryptococcus_sp), Sagen_div (Sagenomella diversispora), Pen_restr (Penicillium restrictum), Exoph_sp (Exophiala_sp), Russ_acri
(Russula acrifolia), Inoc_sp (Inocybe sp), Cenoc_gep (Cenococcum geophilum),
Pen_nod (Penicillium nodositatum), Ino_astr (Inocybe asterospora), Ino_geo (Inocybe
gephylla), Amph_byss (Amphynema byssoides), Tric_terr (Tricholoma terreum). Only
the most abundant and ecologically relevant species are included. Species names are
shown in italics, whereas SHs that were not identified at species level are identified to
the genus level.

Forward selection of explanatory variables identified both autumn temperature (70% of
fitted variation, P=0.002) and precipitation (30% of fitted variation, P=0.002) as
strongly related to fungal community composition across years, together accounting for
6.4% of the total inertia (Fig 2b). When fungal communities were evaluated according
to functional guilds, there was a significant correlation with autumn precipitation and
temperature across years (pseudo-F= 12.6, P<0.001), accounting for 26.7% of the total
inertia. This correlation was generally higher for temperature (82% of fitted variation)
than for precipitation (18% of fitted variation). The overall proportion of amplicons
attributed to mycorrhizal species was higher under colder and drier conditions (Table 1,
Fig. 2b). In contrast, the relative proportion of other functional guilds, such as black
yeasts, moulds and yeasts, were higher under warmer and wetter conditions (Table 1,
Fig 2b). The progressive change across the time span of the study, with free-living fungi
favoured by wet and warm conditions declining in relative abundance, was driven by
particularly warm autumn conditions during 2009 and particularly wet conditions
during 2012 (Fig. 2a). Finally, no effects of precipitation and temperature were
observed on litter saprotrophic taxa (Table 1). All these correlations in community
composition with changes in weather conditions were consistent independently of
whether plots were thinned or not.
**Fig. 2.** CCA species plots showing correlations (a) between relative abundances of fungal SHs and the year identity, and (b) between relative abundances of fungal SHs and the variation in autumn precipitation and temperature. Species symbols in (a) and (b) are coloured according to functional guilds and symbol sizes are proportional to relative abundance.

**Table 1:** Fitting statistics of LME models testing correlations between relative proportions of functional group of soil fungi and inter-annual variation in autumn precipitation and temperature. Significant values are highlighted and (-) denote negative correlations.

<table>
<thead>
<tr>
<th>Functional guilds</th>
<th>Precipitation</th>
<th>Temperature</th>
<th>Precip. × Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>Black yeast</td>
<td>29.37</td>
<td>&lt;0.001</td>
<td>1.08 (-)</td>
</tr>
<tr>
<td>Ectomycorrhizal</td>
<td>20.16 (-)</td>
<td>&lt;0.001</td>
<td>26.78 (-)</td>
</tr>
<tr>
<td>Moss-associated</td>
<td>2.07</td>
<td>0.155</td>
<td>8.8</td>
</tr>
<tr>
<td>Moulds</td>
<td>23.07</td>
<td>&lt;0.001</td>
<td>31.89</td>
</tr>
<tr>
<td>Pathogen</td>
<td>1.99</td>
<td>0.163</td>
<td>58.96</td>
</tr>
<tr>
<td>Root ass. ascomycetes</td>
<td>0.06</td>
<td>0.803</td>
<td>30.07</td>
</tr>
<tr>
<td>Litter saprotroph</td>
<td>0.01 (-)</td>
<td>0.915</td>
<td>1.22</td>
</tr>
<tr>
<td>Soil saprotroph</td>
<td>2.15 (-)</td>
<td>0.147</td>
<td>2.32</td>
</tr>
<tr>
<td>Yeasts</td>
<td>16.94</td>
<td>&lt;0.001</td>
<td>7.96</td>
</tr>
</tbody>
</table>
3.4 Effects on fungal diversity

There was a significant year effect on ectomycorrhizal richness and diversity (for all Hill’s parameters; Fig. 3a, Table 2a), with 27, 34, 35 and 37 species were detected in 2009, 2012, 2013 and 2014, respectively. However, there were no significant changes in diversity (N1, N2) when the entire fungal community (including all functional guilds) was considered, and only marginal changes in richness (N0) were found with time (Fig. S4; Table 2b). Forest thinning did not have a significant effect on fungal richness and diversity (Fig. 3b), nor did the interaction between thinning and year (Table 2b).

**Fig 3.** Hill’s diversity values of the ectomycorrhizal community across the four years considered (a) and across the thinning treatments (b).
Table 2. Year and thinning effects on belowground fungal diversity for the (a) mycorrhizal and the (b) whole fungal community.

<table>
<thead>
<tr>
<th>Effects</th>
<th>(a) Mycorrhizal community</th>
<th>(b) Whole fungal community</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Hill's N0</td>
<td>Hill's N1</td>
</tr>
<tr>
<td>Intercept</td>
<td>1</td>
<td>939.7</td>
</tr>
<tr>
<td>Reads</td>
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<td>66.2</td>
</tr>
<tr>
<td>Thinning intensity</td>
<td>3</td>
<td>0.06</td>
</tr>
<tr>
<td>Year</td>
<td>3</td>
<td>6.42</td>
</tr>
<tr>
<td>Thinning × Year</td>
<td>3</td>
<td>0.99</td>
</tr>
</tbody>
</table>
4. Discussion

We found a thinning-independent directional dynamics of the fungal community within the 5-year study period (Fig. 1a, 1b), related to changes in rainfall and temperature. This directional dynamic seemingly was driven by inter-annual variation in precipitation and temperature (Fig. 2a, 2b). During the warmer autumns early in the study period most non-ectomycorrhizal guilds increased their relative proportions, whereas ectomycorrhizal species were relatively more abundant during the cooler years towards the end of the study period. Moulds and yeasts also increased in abundance during wetter conditions (2012).

4.1 Lack of short-term thinning effects on the fungal community

Forest thinning did neither significantly affect fungal species composition or guild composition, nor fungal diversity. Despite that thinning may have effects on specific fungal species (Bonet et al., 2012; Liu et al., 2016), it seems that most ectomycorrhizal species can survive belowground supported by the remaining trees, seedlings or other ectomycorrhizal plants remaining in the plots (Amaranthus and Perry, 1987; Rosenvald and Lõhmus, 2008). Our results contrast with previously observed effects of more intense timber removal operations, such as clear-cutting, which usually lead to major losses of ectomycorrhizal species (Jones et al., 2003, Parladé et al., 2017), changes in ectomycorrhizal (Varenius et al., 2016, 2017) and/or overall soil fungal community composition (Hartmann et al., 2012; Kyaschenko et al., 2017) and alterations in general soil biology with potential indirect effect on fungal diversity (Colinas et al., 1994a, b). For example, tree harvesting may change soil microclimatic conditions, which in turn may affect soil fungal communities. In this regard, although soil microclimatic data measured in 2013 showed no thinning effects on soil moisture, soil temperature significantly increased in thinned plots (results not shown). Despite these
effects of thinning on soil temperature, thinning effects on belowground fungal diversity and community composition were non-significant. Fungal communities may resist thinning by survival on living roots of retained trees (Varenius et al., 2017) or in naturally established seedlings (Cline et al., 2005). In our study, it seems that the density of remaining trees in all the thinning categories sufficed to act as a post-thinning ‘refuge’ for the mycorrhizal community (Varenius et al. 2017). In addition, other functional guilds, such as saprotrophic fungi, did not respond to thinning either, likely because their substrates were not affected by the thinning operation. Surprisingly, even heavy thinning (up to 70% reduction in stand basal area, down to 350 trees ha\(^{-1}\) left) did not affect the species composition or functional composition of soil fungi, rejecting our hypothesis that extensive thinning would lead to major changes in fungal species composition and dominance of functional guilds. Thus, it seems as relatively sparsely distributed trees may be efficient in preserving the mycorrhizal diversity as long as tree roots and their associated extramatrical mycelium form a continuum across the forest area. Lack of compositional effects of thinning together with climate dependent temporal changes suggest that weather conditions pose a stronger environmental filter on community dynamics that overshadows thinning induced reduction in C flow in this system. One particularity of our study was that thinning was performed with minimal disturbance to avoid soil scarification and compaction (Bonet et al., 2012; using chainsaw rather than heavy machinery to fell the trees). Usually, soil compaction results in a reduction of water retention capacity and it has been shown to affect the soil fungal communities (Hartmann et al., 2012). Perhaps some of the effects of thinning on forest fungi reported in the literature have more to do with soil disturbance than with the actual reduction of basal area. This hypothesis is congruent with our results but it would have to be formally tested in the future.
4.2 Inter-annual changes in community composition and correlation with changes in weather conditions

It could be possible that these directional changes in community composition were driven by changes in temperature, since we observed that temperature was also linearly decreasing across years. These changes in temperature could differently stimulate plant host activity, resulting in distinct fungal responses belowground (Högberg et al., 2010).

In previous studies focused on temperate or boreal forest ecosystems, free-living fungi, such as yeasts, litter saprotrophs or moulds were also found to increase under wetter conditions from an intra-annual perspective (Jumpponen et al., 2010; Voříšková et al., 2013) or under snow cover (Santalahti et al., 2016). In addition, we recently found similar results in Mediterranean forests, also from an intra-annual perspective (Castaño et al., 2018). In our study, yeasts belonging to Tremellomycetes (Cryptococcus and Guêhomyces) decreased in relative abundance during our inter-annual study period, in parallel with decreasing temperature and precipitation, confirming their preference for moist environments (Choudhary and Johri, 2009). In agreement with our third hypothesis, and with Hartmann et al. (2017), Zygomycete fungi (mostly moulds) also responded positively to cooler and wetter climate, but litter saprotrophs did not. Litter-associated fungi accounted for less than 4% of the sequences, probably excluded by our sampling scheme that focused on the humus and mineral horizon. Despite the lack of effects on the moss-associated fungal species found in this study, they were highly represented in wetter years, and intra-annual studies from the same site found a clear positive effect of soil moisture on this group of fungi, both from a spatial and an intra-annual perspective (Castaño et al., 2018). This trend may be related to the phenology of the host trees, as carbon allocation to symbiotic fungi typically peak during the fall (Högberg et al., 2010) and may have shifted with changing weather conditions. The
differential response of fungi in different functional guilds might indicate a competitive advantage for root-associated fungi (i.e. mycorrhizal) over free-living fungi (i.e. yeasts, moulds, moss-associated) under drier conditions. Potentially, hydraulic lift may grant root-associated fungi more resistance to drought, as groundwater can be supplied to fungal symbionts via roots (Allen, 2007; Querejeta et al., 2003, 2017). The decrease in the proportion of free-living fungi under drier conditions may be especially important for Mediterranean forests soils, where water is the most limiting factor for tree growth, and provide insights into future potential climate-change effects on the belowground fungal community and associated processes such as decomposition and nutrient cycling.

5. Conclusions

In our study, thinning did not have a significant effect on fungal community composition or diversity, indicating that these communities are resistant to forest thinning if enough trees are left on site. However, our observations regarding interannual changes in fungal composition and their relationship to changes in meteorological conditions have important implications for our understanding and prediction of future effects of climate change in Mediterranean forests. Further research should address how the climate-related effects on fungal community will affect ecosystem processes such as nutrient cycling, and thinning effects should be evaluated in different tree species and forest ecosystems under different climate regimes.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitivity (MINECO) [grant number AGL2015-66001-C3] and by the Collaborative European project ERANET-INFORMED (PCIN-2014-050). Carles Castaño received the support of the Doctorats Industrials program, funded by the European Union and the European
Social Fund. Sergio de-Miguel was supported by the European Union's Horizon 2020 MultiFUNGtionality Marie Skłodowska-Curie [Grant number IF-EF No-655815]. Josu G. Alday was supported by Ramon y Cajal fellowship [Grant number RYC-2016-20528] and José Antonio Bonet benefited from a Serra-Húnter Fellowship provided by the Generalitat of Catalunya. The authors are very grateful to the PNIN of Poblet for its considerable help with the process of installing and maintaining the experimental plots. We thank Liu Bing, Daniel Oliach, Francesc Bolaño, Jordi Margalef, Josep Miró and Jewel Yurkewich for their assistance with sampling the plots and processing the samples. The constructive comments and suggestions of two anonymous reviewers were improved substantially this manuscript.

**Conflict of interests**

The authors declare no conflict of interests associated with this publication.

**References**


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Fig. S1 (a) Geographical localization of the plots. Each of these plots consisted in two paired plots, one thinned and another one un-thinned, except for the plots 311 and 314 (not included in this study), amounting a total of 24 plots. Map obtained from Google earth V 6.2.2.6613 (November 2017). (c) Thinning design, consisting in three treatment levels (light thinning, medium thinning and heavy thinning) in a 40 × 40 m. plots. (c) Sampling pattern, in which soil samples were obtained in 2009, 2012, 2013 and 2014 in both un-thinned and thinned plots. In total, 8 soil samples were obtained in each of these plots and pooled to a composite sample.
Fig. S2: Principal Response Curves obtained from the whole set of 24 plots.

Thinning intensity effects (Heavy= 50-70% basal area thinned, Medium= 30-50% basal area thinned, Light= <30% basal area thinned, Control= Un-thinned) are tested, and the direction of changes in community composition across years is shown for all thinning intensity levels. Relative proportion increases of specific fungal taxa under certain thinning intensity treatment are shown on the right axis. Although some species became more abundant under specific forest thinning treatments (genera and species list in the right, Y-axis), the overall effects of the thinning treatments were not significant (F=1.6, P= 0.621).
Fig. S3: Principal Response Curves obtained from the whole set of 24 plots. Each curve represents a different year (2009, 2012, 2013, 2014). Here, the factor year is tested, and the direction of changes in community composition across treatments is shown for all years.
Fig. S4 Hill’s diversity values of the whole fungal community across the four thinning treatments (a) and across the four years (b).