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Research paper

Insect defoliation is linked to a decrease in soil ectomycorrhizal biomass and shifts in needle endophytic communities

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Insect outbreaks of increasing frequency and severity in forests are predicted due to climate change. Insect herbivory is known to promote physiological changes in forest trees. However, little is known about whether these plant phenotypic adjustments have cascading effects on tree microbial symbionts such as fungi in roots and foliage. We studied the impact of defoliation by the pine processionary moth in two infested *Pinus nigra* forests through a multilevel sampling of defoliated and non-defoliated trees. We measured tree growth, nutritional status and carbon allocation to chemical defenses. Simultaneously, we analysed the putative impact of defoliation on the needle endophytes and on the soil fungal communities. Higher concentrations of chemical defenses were found in defoliated trees, likely as a response to defoliation; however, no differences in non-structural carbohydrate reserves were found. In parallel to the reductions in tree growth and changes in chemical defenses, we observed shifts in the composition of needle endophytic and soil fungal communities in defoliated trees. Defoliated trees consistently corresponded with a lower biomass of ectomycorrhizal fungi in both sites, and a higher alpha diversity and greater relative abundance of belowground saprotrophs and pathogens. However, ectomycorrhizal alpha diversity was similar between non-defoliated and defoliated trees. Specific needle endophytes in old needles were strongly associated with non-defoliated trees. The potential role of these endophytic fungi in pine resistance should be further investigated. Our study suggests that lower biomass of ectomycorrhizal fungi in defoliated trees might slow down tree recovery since fungal shifts might affect tree-mycorrhizal feedbacks and can potentially influence carbon and nitrogen cycling in forest soils.

Keywords: carbon reserves, chemical defenses, insect outbreak, plant–fungi interactions, plant–herbivore interactions, *Thaumetopoea pityocampa*.

Introduction

Periodic insect outbreaks represent a major disturbance in forest ecosystems, usually causing a sharp decline in tree productivity and growth. Insect outbreaks can also have indirect effects on forest function by altering biotic interactions supported by forest trees. For example, a decline in primary productivity caused by insect defoliation could negatively affect ecosystem processes such as nutrient cycling through affecting belowground communities (Clemmensen et al. 2015, Kristensen et al. 2020, Saravesi et al. 2015). Climate change is expected to increase the severity and frequency of insect outbreaks (Battisti et al. 2005, Hódar and Zamora 2004). For example, warmer conditions can promote the expansion range of some pests to new hosts and areas previously unsuitable for insect development

(Bale et al. 2002, Battisti et al. 2005, Cudmore et al. 2010, Cullingham et al. 2011, Erbilgin 2019, Stenlid and Oliva 2016). Rising temperatures may also alter insect biology by accelerating the developmental rate, increasing reproductive potential and the activity of insect herbivores (Ayres and Lombardero 2000). The impact of insect defoliation on tree physiology and tree-associated microbiota could be contingent on abiotic factors such as drought and temperature (Jacquet et al. 2014). Thus, the effects of insect defoliation on trees and associated microbial symbionts need to be investigated in contrasting climatic environments to be able to predict how these outbreaks will affect the forests in a context of climate change.

Insect herbivores damage conductive structures when feeding on tree stem phloem and reduce the source of available carbon when feeding on needles. Such damage directly influences tree carbon and water economy, as well as biomass and carbon allocation to reserves (Högberg et al. 2001, Oliva et al. 2014). For example, it is known that insect defoliation at tree level reduces primary and secondary growth due to the decrease in photosynthetic leaf area and reduced carbohydrate supply to meristems (Jacquet et al. 2014). In addition, defoliation impact on tree growth is expected to be accentuated by water shortages in drought-prone regions (Jacquet et al. 2014). The impact of insect herbivores on the water and carbon economy of a tree may increase the susceptibility to other stressors, including pathogens (Oliva et al. 2014). In addition, insect herbivore damage triggers the synthesis of chemical and physical defenses in trees that can reduce insect damage and performance (Moreira et al. 2013, 2015). Such defenses are costly, but, if adaptive, they would enhance tree resistance (Eyles et al. 2010, Franceschi et al. 2005).

Recent meta-analysis studies show that insect performance may be challenged by the presence of fungal endophytes (Fernandez-Conradi et al. 2018). It is known that endophytic fungi can play a role in protecting the tree from future attacks (Arnold et al. 2003, Eyles et al. 2010, Raghavendra and Newcombe 2013). Thus, certain endophytes can potentially improve tree immune function against diseases (Arnold et al. 2003, Raghavendra and Newcombe 2013). Similarly, other studies also showed that some of these plant endophytes are recognized as entomopathogenic fungi (Jaber and Ownley 2018). However, the mechanism by which endophytes can protect trees from diseases and insect attacks is still unknown for many fungal taxa. Similarly, whether conifer trees have endophytes that could increase tree resistance against insect defoliators, such as pine processionary moth (PPM), is still unknown.

Several functional guilds of fungi coexist in forest soils, including mycorrhizal, saprotrophs and pathogens, among others (Baldrian 2017). Saprotrophs dominate the upper soil layers and are responsible for the initial litter decomposition (Lindahl et al. 2007), whereas soil pathogens can eventually colonize and attack tree roots. Mycorrhizal fungi are essential organisms for nutrient and water uptake by trees (Mohan et al. 2014, Smith and Read 2008) and, thus, for stress tolerance. Hence, disturbances affecting mycorrhizal fungal communities can alter tree physiological processes including growth or the production of chemical defenses required to defend against biotic and abiotic damage (Karst et al. 2015). Given the mycorrhizal dependency on host plant carbon, insect herbivory feeding may have an indirect negative impact on soil mycorrhizal communities (Högberg et al. 2001, Pec et al. 2016). A decrease in mycorrhizal abundance may also increase the susceptibility of trees to pathogens and can potentially increase saprotrophic activity in litter via 'Gadgil effect' (Sterkenburg et al. 2018). The 'Gadgil effect' is a deceleration of soil carbon cycling mediated by the strong interactions between saprotrophs and ectomycorrhizal fungi competing for resources (Fernandez and Kennedy 2016). It may also be possible that specific fungal groups are more resistant to stresses and, therefore, inconsistent responses to tree defoliation across fungal groups may also occur (Parker et al. 2017). However, it is still unclear whether the changes in patterns of carbon allocation within a tree following a defoliation event have cascading effects on the soil mycorrhizal community and specifically on its biomass. In addition, it is unknown whether defoliation may differently affect each fungal functional group (e.g., saprotrophs, ectomycorrhizal, pathogens).

We studied whether differences in tree growth, tree nutritional status, carbon allocation to storage or chemical defenses in trees defoliated by the PPM (*Thaumetopoea pityocampa* Schiff.) parallel with changes in tree-associated microorganisms such as needle endophytes and soil fungal communities. The PPM is the main defoliator of several Mediterranean conifer species, including *Pinus nigra* (Palacio et al. 2012, Sangüesa-Barreda et al. 2014). The range of *P. nigra* extends over more than 3.5 M ha from North Africa through Southern Europe to the Anatolian plateau, and this species is highly vulnerable to PPM defoliation (Sangüesa-Barreda et al. 2014).

We studied defoliated and non-defoliated trees after a PPM outbreak and, as the outcome of biotic interactions is known to be context dependent, we replicated the study in two environments with contrasting climates and nutrient availability. We hypothesized that (i) defoliated trees will have higher chemical defenses in response to the defoliation; but, because defoliation is expected to reduce the carbohydrate supply to roots, (ii) we expect a reduction in mycorrhizal biomass and an altered soil fungal community structure (abundance, composition and alpha diversity). In addition, (iii) we expect distinct needle endophyte communities in non-defoliated trees as compared to defoliated trees, either due to a recruitment of specific endophytes in response to defoliation or due to specific endophytes increasing tree resistance to defoliation in non-defoliated trees.

Table 1. Tree characteristics of defoliated and non-defoliated *P. nigra* trees growing in Huesca and Teruel.

	Huesca		Teruel	
	Defoliated	Non-defoliated	Defoliated	Non-defoliated
Diameter at breast height (cm)	25.6 ± 4.5	23.7 ± 2.8	14.8 ± 1.7	15.1 ± 1.7
Tree height (m)	9.0 ± 1.4	10.0 ± 1.5	8.7 ± 0.5	8.9 ± 0.5
Defoliation (%)	94.8 ± 2.2	3 ± 6.3	99.3 ± 1.6	2.5 ± 3.5
Tree ring width 2010–2015 (mm)*	1.9 ± 0.4	1.9 ± 0.5	0.4 ± 0.3	0.5 ± 0.3
Tree ring width 2016 (mm)	0.14 ± 0.18	1.68 ± 0.51	0.01 ± 0.01	0.52 ± 0.37
Growth reduction (%) 2016	91.84	–	99.43	–

*Yearly averaged values
Values are means ± SE.
Trees were 50 years old in both sites

Materials and methods

Description of the study sites

We selected two 50-year-old *P. nigra* J.F. Arn. plantations in two climatically contrasting sites located in Aragón (northeast Spain) that were intensively defoliated by the PPM during the large 2015–2016 outbreak in the Pre-Pyrenees region. The PPM is a winter defoliator, and in both sites, defoliation attacks started at the end of 2015 and finished in early spring 2016. In both sites, a small percentage of non-attacked trees coexisted with defoliated neighbors (Table 1, Figures S1 and S2 available as Supplementary Data at *Tree Physiology* Online).

The selected sites greatly differ in terms of climate and productivity. A dry and least productive site is located in Mora de Rubielos (Teruel, 40.190441 N, 0.651928 W, 944 m a.s.l. 491 mm mean annual precipitation, 11.6 °C mean annual temperature) and a wetter site is located in Senegüé (Huesca, 42.557323 N, 0.342423 W, 875 m a.s.l. 808 mm mean annual precipitation, 9.3 °C mean annual temperature) (Table 1).

Tree selection and samplings

At each site, we randomly selected pairs of defoliated and non-defoliated neighboring trees, from which we investigated tree radial growth rates, tree physiology (carbon reserves and chemical defenses), needle-associated fungal communities, soil nutrient availability and the soil fungal communities. Since susceptibility to defoliation may depend on the position of the trees in the landscape (Régolini et al. 2014), defoliated and non-defoliated trees were sampled inside the forest to avoid any edge effect (>∼10 m.). We performed a paired sampling approach (e.g., each defoliated tree had its non-defoliated tree pair at <10 m) to reduce confounding spatial effects. At each site, 10 non-defoliated (i.e., none or negligible defoliation) and 10 severely defoliated (i.e., >90% defoliation) trees were selected within an area of ca. 2000 m^2 (20 pairs, 10 pairs per site). Tree selection was performed in February 2016, when the outbreak finished. At that time, crown defoliation and the number of PPM colony nests per tree were determined and used as proxies for infestation intensity (see Supporting Information Methods S1 available as Supplementary Data at *Tree Physiology* Online). Samples for carbon reserves and chemical defenses were taken during May 2016. Soil samples for the analyses of the fungal community and biomass were taken during May 2016 and again in July 2016. Needle samples to study the needle-associated fungal communities were taken in July 2016. Increment core samples were taken in September 2016.

Tree radial growth

Dendrochronological methods were used to assess the radial growth of all the trees over the past 50 years. Two increment wood cores from each tree were extracted at breast height (1.3 m) using a 5-mm Pressler increment borer. Wood cores were air-dried before carefully sanding their surface until tree-ring boundaries were clearly visible. Tree rings were visually cross-dated, and their width was measured to the nearest 0.01 mm using a binocular microscope and a LINTAB measuring device (Rinntech, Heidelberg, Germany). The visual cross-dating was checked using the COFECHA program, which calculated correlations between each individual series (i.e., the mean of the two radial cores) with the mean series of each group of trees at each site (Holmes 1983).

Tree non-structural carbohydrate reserves

We used non-structural carbohydrates (NSCs) in the wood as a proxy for tree carbon reserves. To quantify the concentration of NSCs, a sapwood sample was obtained from each tree at a height of 1.3 m using a 12-mm Pressler increment borer (Figure S2 available as Supplementary Data at *Tree Physiology* Online). Wood samples were transported to the laboratory at ca 4 °C and then stored at −20 °C until freeze-dried. Wood samples were then weighed and milled to a fine powder using a ball mill. Soluble sugars were extracted with 80% (v/v) ethanol and their concentration was colorimetrically determined using the phenol-sulfuric method (Buysse and Merckx 1993). Remaining starch and complex sugars were enzymatically digested with an enzyme mixture containing amyloglucosidase to reduce glucose,

as described in Palacio et al. (2012). NSCs measured after ethanol extractions were regarded as soluble sugars, whereas carbohydrates measured after enzymatic digestion were considered to be starch. NSC concentrations were determined by calculating the sum of soluble sugar and starch concentrations in mg g^{-1} of dry matter.

Tree chemical defenses

As a functional proxy of the defensive status of each tree, we measured the resin flow of all trees following a procedure similar to Lombardero et al. (2000). We removed a disc of bark (13 mm in diameter, including phloem and cortex) on opposite sides of each tree at breast height, and a polyethylene device designed for resin collection was fitted to each hole and gently attached to the tree bole (Figure S2 available as Supplementary Data at *Tree Physiology* Online). Pre-weighed 15-ml conical centrifuge plastic tubes (falcon tubes, Corning, USA) were screwed into the plastic devices to collect the flowing resin. Phloem plugs were immediately placed in an ice cooler (4 °C), transported to the lab and frozen within 4 h for further analyses. Falcon tubes were removed after 24 h, immediately capped, transported to the laboratory at 4 °C and weighed to estimate the mass of resin flowing out.

Terpenes were extracted from the phloem plugs and analysed by gas chromatography following the method described by López-Goldar et al. (2019) with some modifications (see Supporting Information Methods S1 available as Supplementary Data at *Tree Physiology* Online). Identified compounds were grouped by their nature and the concentration of the total mono-, sesqui- and di-terpene fractions were used for statistical analyses.

Chemical defenses in the canopy were assessed by determining the concentration of non-volatile resin (the diterpene fraction), total polyphenolics and condensed tannins in both the current-year needles and stem sections of terminal branches following Sampedro et al. (2011) (see Supporting Information Methods S1 available as Supplementary Data at *Tree Physiology* Online).

Soil sampling and determination of macronutrients in soil and needles

A cylindrical soil core (12-cm deep and 5 cm in diameter) was collected systematically 1 m from the trunk below each tree following the four cardinal directions (N, S, E and W; four soil samples per tree). The upper layer of the soil (<3 cm) of needles or partially degraded debris was removed before sampling (Castaño, Alday, et al. 2018). Collected soil samples were stored at 4 °C and sieved through a 3-mm mesh within 24 h. The four soil samples from each tree were pooled to obtain a single composite sample. The composite sample was then freeze-dried and ground to a fine powder using a sterile mortar and a pestle. Available nitrogen and phosphorus content were analysed in the soil and total nitrogen and phosphorus in the needles (see Supporting Information Methods S1 available as Supplementary Data at *Tree Physiology* Online).

Soil fungal biomass

The total soil fungal biomass was estimated by quantifying the fungal-specific biomarker ergosterol (Nylund and Wallander 1992). Ergosterol concentration in pentane extracts was determined by HPLC as described by Hagenbo et al. (2017), with some modifications (see Supporting Information Methods S1 available as Supplementary Data at *Tree Physiology* Online).

Soil fungal community analysis

DNA was extracted from 500 mg of ground soil using the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol. Fungal DNA was PCR-amplified in a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA) using primers gITS7 (Ihrmark et al. 2012) and ITS4 (White et al. 1990) following Castaño, Lindahl, et al. (2018), with some modifications (see Supporting Information Methods S1 available as Supplementary Data at *Tree Physiology* Online). The resulting fungal DNA library was sequenced using the Illumina MiSeq 2 × 300 bp platform, generating 12.5 million sequences.

Needle-associated fungal community

New needles (needles that grew after the defoliation event, i.e., less than 1-year-old needles) and old needles (needles that grew before the defoliation event, i.e., 2-year-old needles) of non-defoliated and defoliated trees were sampled. Two branch tips were cut from each tree and 10 new and 10 old needles were collected from each branch. Needles were immediately transported to the lab in ice coolers, frozen and stored at −20 °C until processed. Needles were washed with 0.1% Tween-20 solution to remove fungi on the needle surface, rinsed with sterile water, freeze-dried for 48 h, and ground to a fine powder.

Fungal DNA within 100 mg of needles was extracted using a NucleoSpin® Plant II kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol. Fungal DNA amplifications and library preparations were carried out following the same methodology as that described in the 'Soil fungal community analysis' section; however, primer fITS7 (Ihrmark et al. 2012) was used instead of gITS7 to avoid co-amplification of plant DNA. The DNA library was sequenced using the Illumina MiSeq platform, with 300-bp paired-end read lengths, generating 12 million sequences.

Bioinformatic analyses

Both soil and needle fungal libraries were separately screened for quality control and sequence clustering using the SCATA pipeline (https://scata.mykopat.slu.se/). Internal transcribed spacer sequences with <200 bp were removed, and remaining sequences were screened for primers (requiring 90% primer match) and sample tags. Sequences were also quality filtered,

removing data with an average quality score of <20 or with a score of <10 at any position, using the amplicon quality option. Sequences were pair-wise compared using 'usearch' (Edgar 2011) after collapsing homopolymers to 3 bp. Pairwise alignments were scored using a mismatch penalty of 1, a gap open penalty of 0 and a gap extension penalty of 1. We used the species hypothesis (SH) concept to cluster the sequences (Kõljalg et al. 2013) using a single linkage clustering method, requiring a maximum distance of 1.5% to the closest neighbor to enter clusters. SHs found in negative controls were excluded from further analysis, together with singletons and sequences with tag jumps. Sequence data are archived in NCBI's Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under accession number PRJNA564427. Sequencing data, fungal identifications and environmental data can be downloaded in Mendeley data, doi: 10.17632/hg8ncsyhkt.1.

Taxonomic and functional identification

We taxonomically identified the 400 most abundant SHs of the soil fungal community, and the 180 most abundant SHs of the needle community, which represented more than 93% of the total sequences for each data set. The most abundant sequences from each SH were selected for taxonomic identification, using massBLASTer implemented in PlutoF against the UNITE database (Abarenkov et al. 2010). Taxonomic identities at species level were assigned using >98.5% similarity. Functional identification was determined using FUNGuild (Nguyen et al. 2016). Exploration types of the ectomycorrhizal species were assigned according to Suz et al. (2014) and the dEEMY database (Agerer and Rambold 2017).

Statistical analyses

Differences between defoliated and non-defoliated trees in terms of tree growth, soil fungal biomass, carbon reserves and chemical defenses were analysed using linear mixed-effects models using the 'nlme' package (Pinheiro et al. 2016). In these models, 'defoliation', 'site' and the 'defoliation × site' interaction were defined as fixed factors, whereas identity of each pair of trees (defoliated and non-defoliated) was defined as a random effect. Residuals of these models were checked to meet the requirements of normality, whereas heterogeneous residual variance models were fitted when deviations from homoscedasticity were detected. Dependent variables were square root or log transformed to achieve normality when needed. All statistical analyses were implemented in the R software environment (v3.0.2 version; R Core Team 2015).

An overall analysis of the fungal community composition in soil and needles was carried out using Permutational multivariate analysis of variance based on distance matrices (using the function 'adonis' and Bray–Curtis distances with the 'vegan' package (Oksanen et al. 2015). For the soil fungal community, the 'defoliation × site' interaction was defined as the explanatory factor. For the needle-associated fungal community, the 'defoliation × needle age' interaction was also considered, being 'needle age' a factor with two levels, 'old' referring to needles present before the outbreak, and 'new' referring to needles emerging the year after the outbreak. Effects of these factors were tested using Monte Carlo, running 999 permutations under the full model. These analyses were carried out separately (i) using the Hellinger transformed operational taxonomic unit (OTU) table and (ii) the Hellinger transformed table of the functional guilds as a response variable. Subsequently, non-metric multidimensional scaling was used to visualize separately the soil and the needle fungal community differences using Bray–Curtis distances. Changes in the relative abundance of functional guilds and exploration types in response to defoliation were assessed by LME to eliminate any potential effect caused by the spatial proximity of trees. In these models, the defoliation × site interaction was defined as a fixed factor, and the identity of each pair of trees was defined as a random effect.

Hill's series of diversity indices were used to compare differences in diversity values between non-defoliated and defoliated trees in terms of their associated soil and needle fungal communities. Separate diversity analyses were also carried out considering only species belonging to specific functional groups (e.g., ectomycorrhizal or saprotrophs). 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. N0, N1 and N2 were calculated from asymptotic estimates implemented in the 'iNEXT' R package (Hsieh et al. 2016) and curves were obtained from the derived value obtained from each number of reads. The diversity of soil fungal communities under non-defoliated and defoliated trees was compared by LME, including the 'defoliation × site interaction', whereas significant effects affecting the needle-associated fungal community that were caused by the defoliation event were studied by analysing the 'defoliation × needle age' interaction. Identity of each pairs of trees was again defined as a random effect.

Finally, we assessed whether a particular OTU could be identified as an indicator species of soil- or needle-associated fungal communities of either non-defoliated or defoliated trees using Species Indicator Index implemented in the package 'indicspecies' (De Cáceres and Legendre 2009). Here, we also analysed the relative abundance of each OTU as a way of considering the bias caused by the different sequencing depths. We used the function 'multipatt' together with the parameter IndVal.g to manage the unequal group sizes.

Results

Radial growth, carbon reserves and chemical defenses in defoliated and non-defoliated trees

Defoliation had large consequences in most of the assessed traits, although responses also appeared to be site specific.

Trees largely differed in terms of their growth, carbon reserves and chemical defenses depending on the site (Table S1 available as Supplementary Data at *Tree Physiology* Online). Trees growing in Teruel showed reduced growth rate, smaller NSC reserves (Table S1 available as Supplementary Data at *Tree Physiology* Online) and produced fewer chemical defenses than trees growing in Huesca (Figures S3 and S4 available as Supplementary Data at *Tree Physiology* Online).

Defoliated and non-defoliated trees did not differ in tree-ring width in the years before the outbreak (Table S1 available as Supplementary Data at *Tree Physiology* Online; $F_{[1,18]} = 0.6$; $P = 0.459$). However, tree-ring width of defoliated trees after the outbreak was 90% smaller than those of non-defoliated trees (Table 1, Table S1 available as Supplementary Data at *Tree Physiology* Online). The 'defoliation × site' interaction did not have a significant effect on radial growth (Table 1). Furthermore, sapwood NSC concentration did not significantly differ between defoliated and non-defoliated trees (Table S1 available as Supplementary Data at *Tree Physiology* Online). Concentration of monoterpenes ($P = 0.014$), sesquiterpenes ($P = 0.006$) and diterpenes ($P = 0.019$) in the phloem of the main bole were 25–30% higher in defoliated than in non-defoliated trees in Teruel, but no differences were found in Huesca (Figure S3 available as Supplementary Data at *Tree Physiology* Online). However, the concentration of non-volatile resin (mainly diterpenes) in the needles of defoliated trees was significantly greater than those of non-defoliated trees in Huesca, whereas the opposite pattern was found in the terminal branches (30% lower non-volatile resin concentration in defoliated trees than in non-defoliated trees). In Huesca but not in Teruel, the concentration of polyphenols was significantly higher in branches of defoliated trees than in non-defoliated trees (15%; Figure S4 available as Supplementary Data at *Tree Physiology* Online). We did not find significant differences in resin flow between defoliated and non-defoliated trees across both sites (Table S1 and Figure S4 available as Supplementary Data at *Tree Physiology* Online).

Nitrogen and phosphorus in soils and needles

Overall, we found no significant differences in the concentration of soil mineral nitrogen and phosphorus between defoliated and non-defoliated trees (Table S2 and Figure S5 available as Supplementary Data at *Tree Physiology* Online). However, the 'site × defoliation' interaction showed a significant and opposite pattern across sites for nitrogen. Thus, the concentration of soil nitrogen was significantly greater below defoliated trees than below non-defoliated trees in Huesca, while the opposite pattern was observed in Teruel (Table S2 and Figure S5 available as Supplementary Data at *Tree Physiology* Online).

Regarding the tree nutritional status, we found differences on both the nitrogen and phosphorus content in needles between defoliated and non-defoliated trees, with significantly higher nutrient concentrations in defoliated than in non-defoliated trees in Huesca (Supporting Information Table S3 and Figure S6 available as Supplementary Data at *Tree Physiology* Online). In the case of phosphorus concentration, there was a significant 'site × defoliation' interaction, as differences between defoliated and non-defoliated trees were strong in Huesca but not evident in Teruel (Table S3 and Figure S6 available as Supplementary Data at *Tree Physiology* Online).

Soil fungal community

There was less soil fungal biomass below defoliated trees than below non-defoliated trees ($P = 0.004$), irrespective of the site ('site × defoliation' $P = 0.288$). A less fungal biomass in the soil under defoliated trees was especially evident in Huesca, with a 31% reduction ($P = 0.028$; Figure S7 available as Supplementary Data at *Tree Physiology* Online); however, differences were less marked in Teruel, where soil fungal biomass was 17% lower under defoliated trees ($P = 0.049$; Figure S7 available as Supplementary Data at *Tree Physiology* Online).

Soil fungal community composition differed between sites ($P < 0.001$, $R^2 = 0.33$) and between defoliated and non-defoliated trees ($P = 0.026$, $R^2 = 0.04$; Figure 1); however, we did not find a significant 'site × defoliation' interaction ($P = 0.089$, $R^2 = 0.03$). Accordingly, there were significant differences in the relative abundance of soil fungal ecological guilds under defoliated and non-defoliated trees at both sites ($P = 0.002$, $R^2 = 0.16$). The relative abundance of ectomycorrhizal species was about 15% lower in soils below defoliated trees than in non-defoliated trees (Figure 2a, Table S4 available as Supplementary Data at *Tree Physiology* Online). Several ectomycorrhizal species such as *Rhizopogon roseolus*, *Thelephora* spp., *Tricholoma terreum* or taxa belonging to *Inocybe* spp. were more abundant below non-defoliated trees. In contrast, higher relative proportions of other fungal ecological guilds, including pathogens and saprotrophs, were detected under defoliated trees than under non-defoliated trees (Figure 2d; Table S4 available as Supplementary Data at *Tree Physiology* Online). No general differences in terms of exploration types of fungi were found between defoliated and non-defoliated trees (Table S4 available as Supplementary Data at *Tree Physiology* Online). However, a significant 'site × defoliation' interaction was found for species with short ($P = 0.042$) and long exploration types ($P = 0.025$; Figure 2e), with lower abundance of long exploration types and higher abundance of short exploration types in defoliated trees in Huesca.

Soil fungal alpha diversity indices (Hill's N0, N1 and N2) were higher under defoliated than under non-defoliated trees at both sites (Table S4 available as Supplementary Data at *Tree Physiology* Online, Figure 3). When analysed according to the fungal functional groups, free-living species (i.e., saprotrophs and pathogens) were more diverse under defoliated trees than under non-defoliated trees ($P < 0.001$, Table S4 available as Supplementary Data at *Tree Physiology* Online). However, alpha diversity of ectomycorrhizal species under defoliated trees did

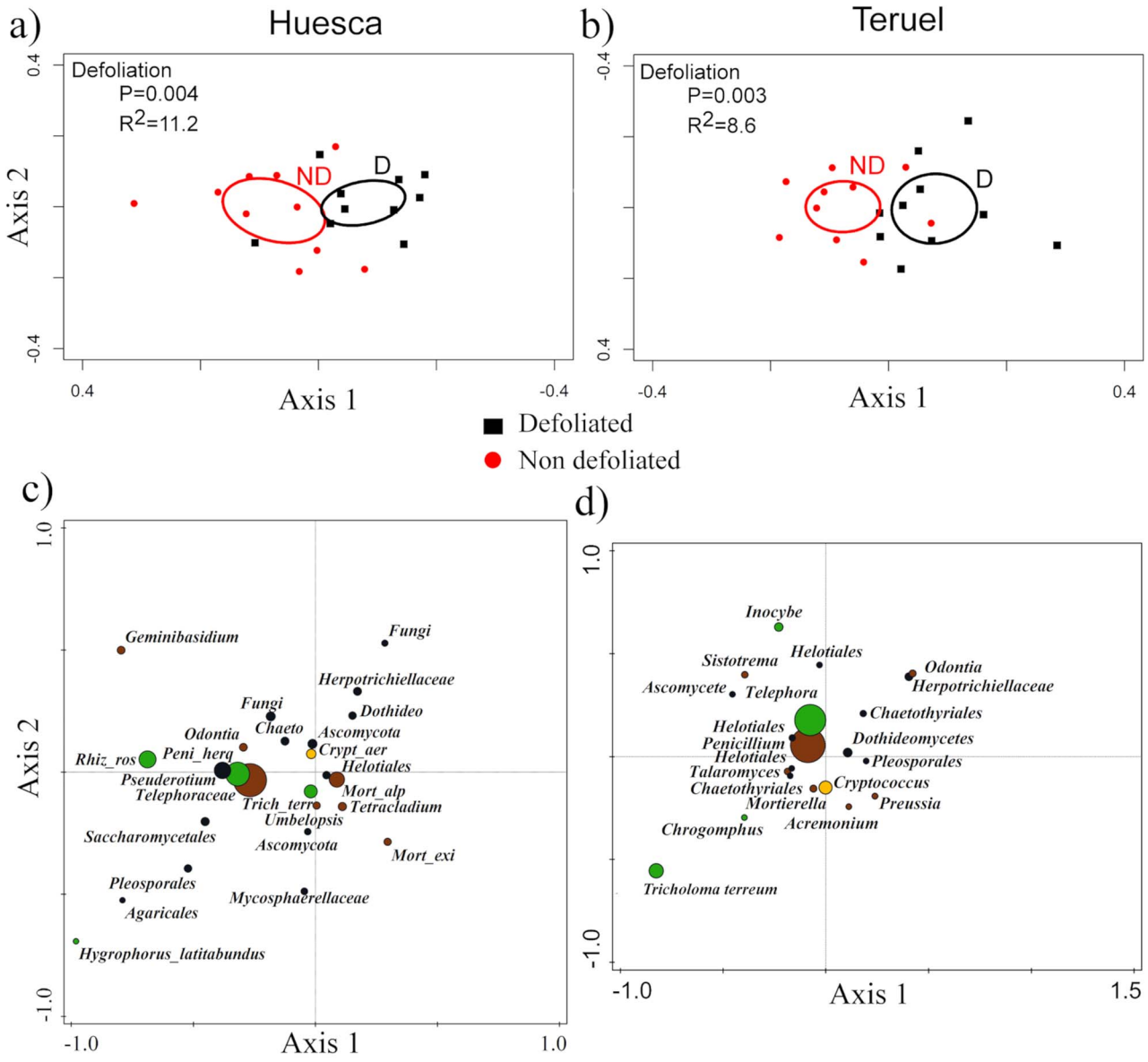

Figure 1. Fungal communities detected in soils under defoliated (D) and non-defoliated (ND) *P. nigra* trees. Nonmetric multidimensional scaling of the soil fungal community under defoliated and non-defoliated trees in the two study sites, Huesca (a) and Teruel (b) sites. Ellipses represent the 95% interval confidence for each site. Each black square or red circle represents the soil under a defoliated or non-defoliated tree, respectively. Plots (c) and (d) represent fungal OTUs detected in Huesca and Teruel, respectively, with each taxon colored according to their functional group (ectomycorrhizal fungi, green; saprotrophs, brown; yeasts, yellow; unknown, black). OTU abbreviations: *Chaeto = Chaetothyriales; Crypt_aer = Cryptococcus aureus; Dothideo = Dothideomycetes*; *Mort_alp = Mortierella alpina; Mort_exi = Mortierella exigua; Peni_herq = Penicillium herquei*; *Rhiz_ros = Rhizopogon roseolus*; *Trich_terr = Tricholoma terreum.*

not differ ($P > 0.05$, Table S5 available as Supplementary Data at *Tree Physiology* Online), except for richness, which was slightly higher in defoliated trees than in non-defoliated trees.

Needle-associated fungal community

Needle-associated fungal communities of defoliated and non-defoliated trees differed significantly ($P < 0.001$, $R^2 = 0.05$; Figure 4), and there was a significant 'site × defoliation' interaction ($P < 0.001$, $R^2 = 0.05$). The 'needle age × defoliation' interaction was significant ($P = 0.014$, $R^2 = 0.02$), indicating that differences in the fungal community between defoliated and non-defoliated trees differed between new and old needles (i.e., there was a defoliation effect; Figure 4). Differences between sites were greater ($P < 0.001$, $R^2 = 0.11$) than the differences observed between defoliated and non-defoliated trees (Figure 4).

The composition of ecological guilds of fungi between old and new needles was significantly different ($P < 0.001$, $R^2 = 0.09$); however, no significant differences were found between defoliated and non-defoliated trees ($P > 0.05$). Fungal

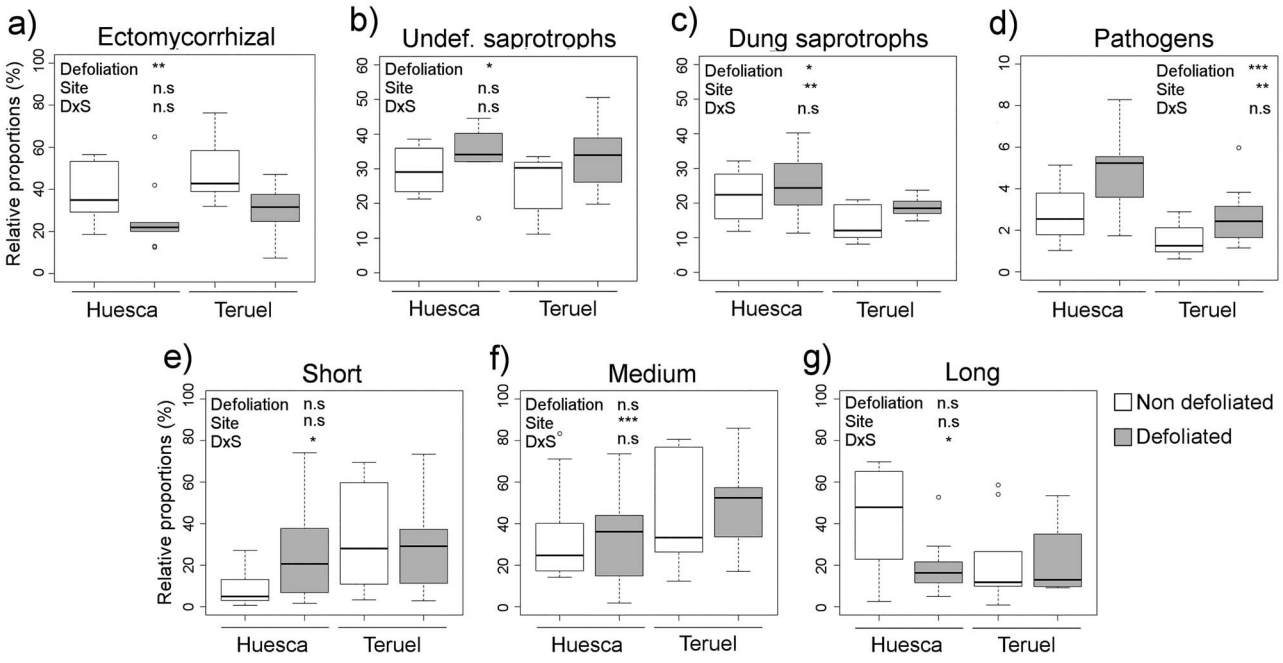

Figure 2. Relative abundance of the four most abundant soil fungal trophic guilds (following Nguyen et al. 2016; a–d) and of three functional mycorrhizal exploration types (e–g) in the soil under defoliated (gray box plots) and non-defoliated (white box plots) *P. nigra* trees in Hueca and Teruel. Circles represent observational values outside the interquartile ranges, whereas asterisks indicate significant differences between defoliated and non-defoliated trees (significance levels: '***' $P < 0.001$, '**' $P < 0.01$, '*' $P < 0.05$, '·' $P < 0.1$, n.s. non-significant). Different scales are used for some of the functional groups in order to visualize their abundance. Undef. Saprotrophs are undefined saprotrophs that are considered either saprotrophs with unknown specific niche or function or generalist saprotrophs.

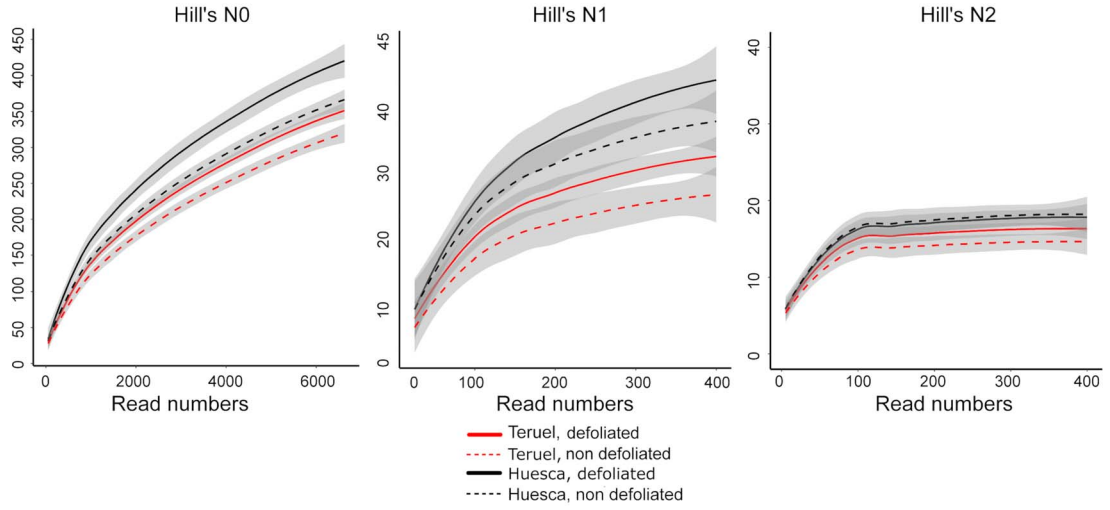

Figure 3. Interpolated OTU-accumulation curves based on internal transcribed spacer read numbers from soil under non-defoliated (red lines) and defoliated *P. nigra* trees (black lines) in Huesca (continuous lines) and Teruel (dashed lines) study sites. Three Hill's indices are shown: (a) Chao1 richness N0, (b) an anti-logarithm of Shannon's diversity index N1 and (c) the inverse Simpson diversity index N2. *X*-axes represent ITS2 read counts whereas *y*-axes represent Hill's values.

alpha diversity, measured by Hill's N1 and N2 but not fungal richness N0, showed a significant 'needle age × defoliation' interaction (Richness N0: $P = 0.874$, N1: $P = 0.007$, N2: $P = 0.011$). Thus, the fungal alpha diversity within new needles from defoliated trees was greater than that within new needles from non-defoliated trees (Figure 5), corresponding with increasing needle nutrients in new needles from defoliated trees (nitrogen and phosphorus). Specifically, indicator-species analyses revealed that *Cenangium ferruginosum* was strongly associated with old needles of non-defoliated trees and

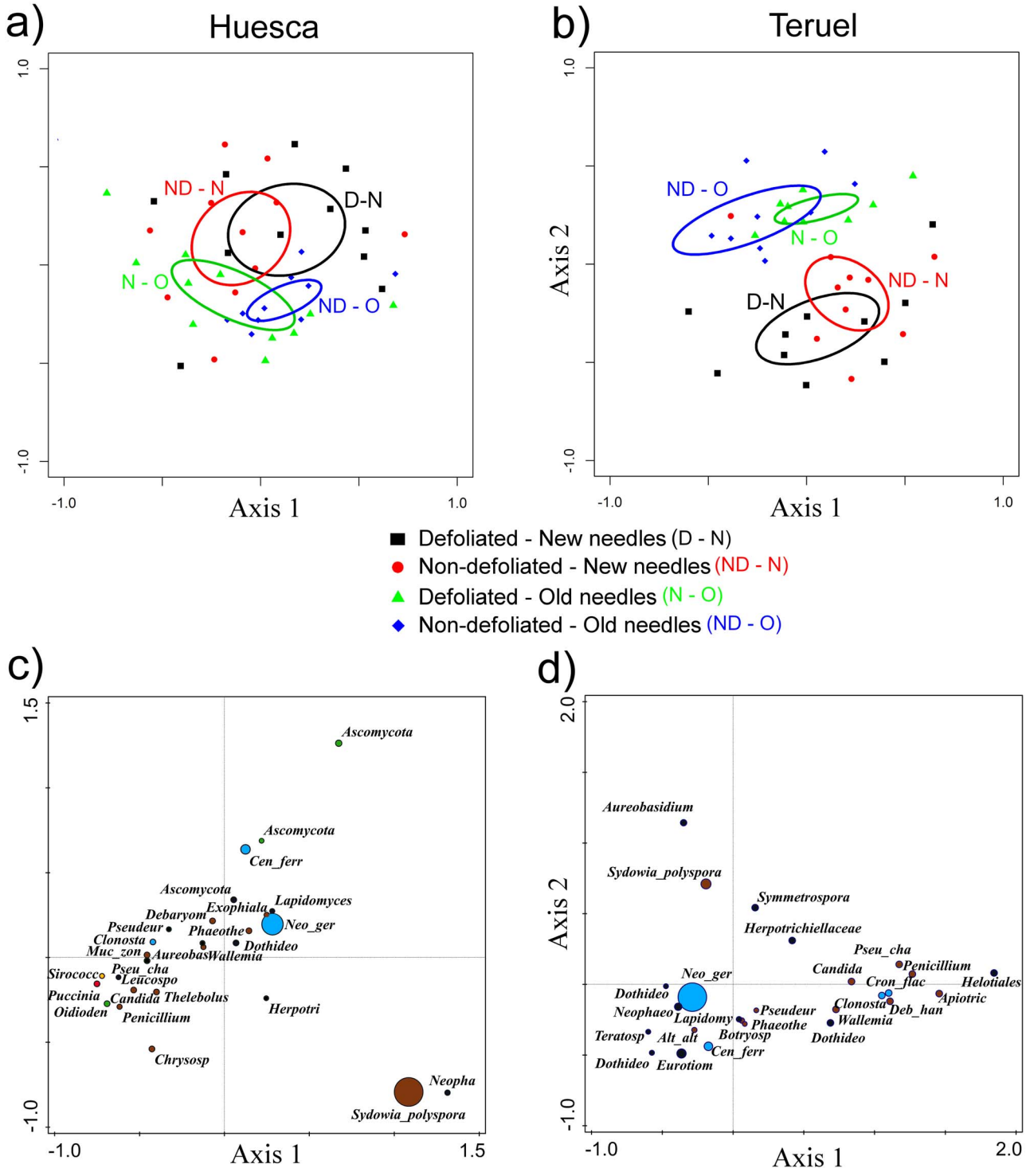

Figure 4. Needle-associated fungal communities detected in needles of defoliated (D) and non-defoliated (ND) *P. nigra* trees. Nonmetric multidimensional scaling of needle-associated fungal communities showing the differences in fungal composition between defoliated and non-defoliated trees and new and old needles in Huesca (a) and Teruel (b) study sites. Each black square represents fungi in new needles of defoliated trees; red circles represent fungi in new needles of non-defoliated trees; green triangles represent fungi in old needles of defoliated trees and blue diamonds represent fungi in old needles of non-defoliated trees. Ellipses represent the 95% interval confidence for each site. Plots (c) and (d) represent the species plot for fungi detected in Huesca and Teruel sites, respectively, with each taxon colored according to their functional group (pathogens, light blue; saprotrophs, brown; yeasts, yellow; ectomycorrhizals, green; unknown, black). OTU abbreviations: *Alt_alt* = *Alternaria alternata*; *Botryosp* = *Botryosphaeriales*; Cen_ferr = *Cenangium ferruginosum*; *Clonosta* = *Clonostachys*; Cron_flac = *Cronartium flaccidum*; *Deb_han* = *Debaryomyces hansenii*; *Dothideo* = *Dothideomycetes*; *Eurotiom* = *Eurotiomycetes*; *Lapidomy* = *Lapidomyces*; *Muc_zon* = *Mucor zonatus*; Neo_ger = *Neocatenulostroma germanicum*; *Neopha* = *Neophaeomoniella*; *Oidioden* = *Oidiodendron*; *Phaeothe*, = *Phaeothecoidea*; *Pseudeur* = *Pseudeurotium*; Pseu_cha = *Pseudopithomyces chartarum*; *Sirococc* = *Sirococcus*; *Teratosp* = Teratosphaeriaceae.

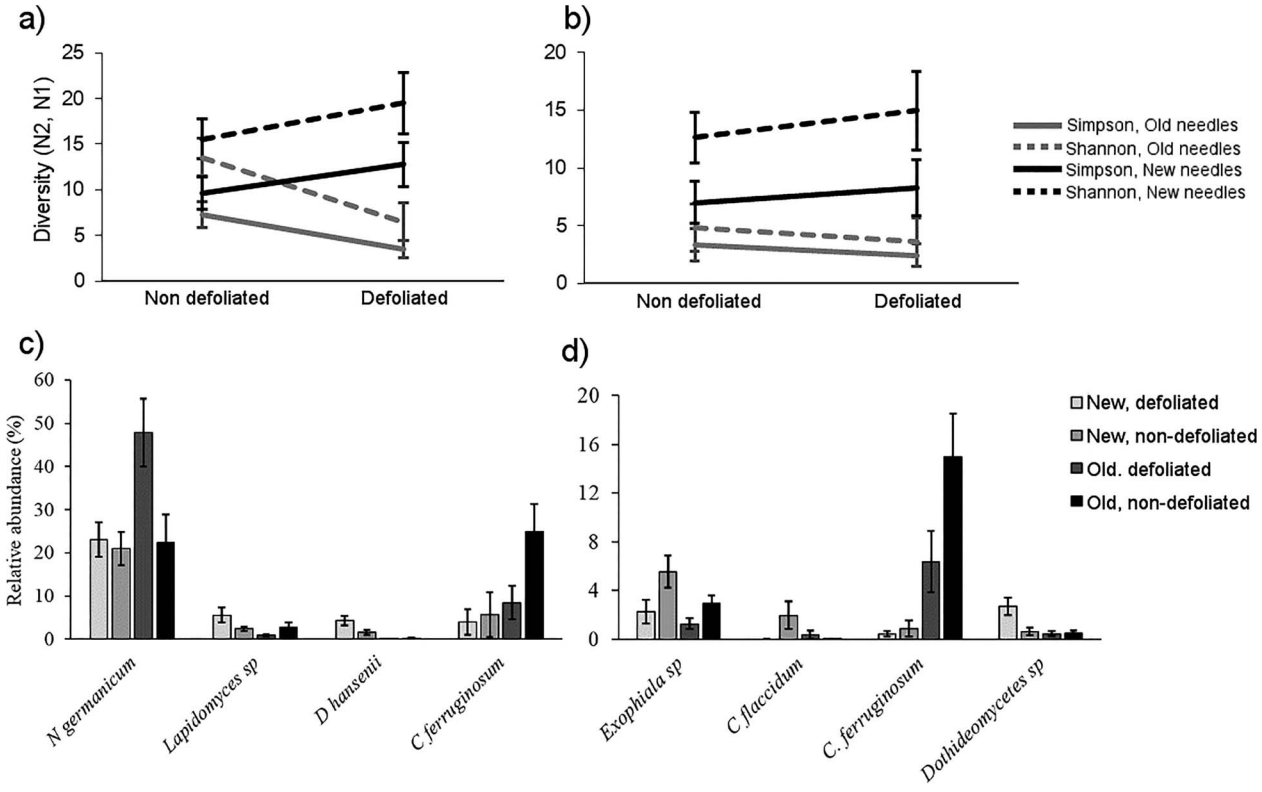

Figure 5. Interactive effects of defoliation and needle age on the diversity of the aboveground fungal community. In Huesca (a) and Teruel (b) study sites, we considered the Hill's diversity indices N1 and N2 of non-defoliated and defoliated *P. nigra* trees. In plots (a) and (b) black lines refer to new needles (i.e., formed after the defoliation event), whereas gray lines refer to old needles (i.e., formed before the defoliation event). Continuous lines indicate N1 Hill's numbers, whereas dotted lines indicate N2 Hill's numbers (values are means ± SE). The indicator species that were significantly affected by pine defoliation in Huesca and Teruel are plotted in (c) and (d), respectively. Only fungal taxa identified down to at least at genus level and detected more than five times are shown. Taxa abbreviations: *N germanicum* = *Neocatenulostroma germanicum*; *D hansenii* = *Debaryomyces hansenii*; *C. ferruginosum* = *Cenangium ferruginosum*; *C flaccidum* = *Cronartium flaccidum*.

Neocatenulostroma germanicum was associated with old needles of defoliated trees (Figure 5).

Discussion

The defoliation probably leads to an increase in the production of some chemical defenses, although these responses seem to be site specific. Terpenes are used by conifers as a defense against insects (Keeling and Bohlmann 2006, Mumm and Hilker 2006) and are often synthetized upon biotic challenge (Karban 2011) to avoid continuous production of C-costly chemical defenses when they are not needed (Gershenzon 1994). However, we found no evidence of a systemic anti-herbivore response based on increased allocation to the full defensive range of chemical compounds across the different tissues of defoliated trees. Three non-exclusive processes may explain this effect: (i) trees mobilize C from other tissues than to the damaged area to construct defenses, (ii) damage may trigger specific defense responses based on particular chemical compounds instead of leading to a generalized allocation of C to defense all over the tree or (iii) PPM caterpillar effects on tree immune response may prevent tree defense induction, as found in other moth–plant interactions (Moreira et al. 2013). However, evidence of a systemic response to defoliation by PPM has been reported in other studies (Achotegui-Castells et al. 2013, Rivas-Ubach et al. 2015). By contrast, Moreira et al. (2013) reported in saplings a strong response in distal tissues (main stem) in terms of phenolics but not in those tissues targeted by the processionary moth. However, in our study, we did not find a clear difference in phenolic abundance between defoliated and non-defoliated trees.

Neither a smaller growth rate nor an induced defense of specific chemical defenses following defoliation seemed to have an impact on NSC reserves. Previous findings also reported that reduced tree growth after defoliation is not necessarily accompanied by a decrease in NSC concentrations (Wiley et al. 2013). Our results are consistent with the previous findings in which defoliation by PPM was not associated with changes in NSC concentrations in sapwood and needles (Palacio et al. 2012). By contrast, NSC depletion has been observed for other types of stressors such as drought or root pathogens (Camarero et al. 2015, Galiano et al. 2011). Perhaps insect herbivory

damage on tissues that act as both a source and sink of carbon resources (i.e., foliage) does not trigger shifts in carbon reserves because a reduced carbon assimilation is counteracted by smaller needs of assimilated carbon (Brodde et al. 2019).

Our findings agree with previously described cascading effects of reduced carbon assimilation by trees affecting belowground mutualists (Högberg et al. 2001, Kristensen et al. 2020, Pec et al. 2016). In our study, insect defoliation was linked to changes in the soil fungal community structure and trophic function. The ectomycorrhizal community was the most affected, with an overall lower ectomycorrhizal abundance in soils under defoliated trees than under non-defoliated trees. Our findings are in line with other related studies in which the mycorrhizal community is negatively affected by other disturbances such as beetle attacks (Ŝtursova et al. 2014), forest clear-cuttings (Kohout et al. 2018) or tree girdling (Högberg et al. 2001). However, although fungal biomass decreased in defoliated trees, we did not observe significant shifts in terms of ectomycorrhizal alpha diversity, indicating that defoliation only reduced soil mycorrhizal biomass. This finding supports the hypothesis that ectomycorrhizal fungal composition and alpha diversity may not be affected if enough trees remain alive in the stands after a disturbance (Castaño, Alday, et al. 2018), which is often the case after PPM outbreaks. In contrast, a decrease in ectomycorrhizal alpha diversity has been consistently reported after bark beetle outbreaks (Pec et al. 2016, Saravesi et al. 2015, Ŝtursova et al. 2014). In contrast to ectomycorrhizal fungi, we observed an increase in the relative abundance and alpha diversity of other soil fungal functional groups (i.e., saprotrophs and pathogens) under defoliated trees compared to non-defoliated. Many opportunistic saprotrophic fungi, such as *Penicillium* or *Mortierella* (Brabcová et al. 2016), can potentially feed on dead mycelium from mycorrhizal species but can also profit on dead organic matter or dying roots. In addition, molds were previously found to be positively associated with mycorrhizal mycelium turnover (Castaño, Lindahl, et al. 2018, Sterkenburg et al. 2018). The loss of soil mycorrhizal biomass may impair the capacity of trees to recover from defoliation and temporarily increase their susceptibility to biotic and abiotic stressors but could also stimulate saprotrophs by the 'Gadgil' effect (Sterkenburg et al. 2018), resulting in an increase in the decomposition of litter owing to the supression of mycorrhizal fungi.

We observed shifts in the needle-associated fungal community following defoliation. Fungal communities in newly developed shoots from defoliated trees were different from those that developed in non-defoliated trees; however, the changes in the fungal communities were low and not as prominent as those detected in soil communities, as shown by the low R^2 values of the multivariate analyses. Simultaneously, the defoliation increased differences in needle endophyte communities between old and new needles. Our results suggest that differences in needle fungal community composition between defoliated and non-defoliated trees may be unrelated to needle nutritional status. Differences in endophytic community composition were reduced in the site where the impact defoliation on needle nutrient concentration was the least. This fact is interesting and deserves further research. Interestingly, the needle fungal alpha diversity patterns correlated with the needle nutrient content patterns (nitrogen, phosphorus), with both higher fungal alpha diversity and higher nutrient levels in new needles from defoliated trees than in new needles from non-defoliated trees. These changes could be a response to the defoliation, but whether such response may be to prevent future attacks needs to be further explored. We found in both locations that *C. ferruginosum*, a weak plant pathogen, was consistently more abundant in old needles (i.e., likely reflecting the pre-defoliation community) from non-defoliated trees. Foliar endophytes can potentially increase host fitness and resistance against biotic and abiotic stresses (Redman et al. 2002), improving tree immune function against diseases (Arnold et al. 2003, Raghavendra and Newcombe 2013). Similarly, it has been shown that certain endophytes may decrease insect performance (Fernandez-Conradi et al. 2018). However, based on our results, we are not able to fully decipher the role played by endophytes such as *C. ferruginosum* in modulating PPM performance. Thus, further research is needed to investigate how these fungi may interact with this relevant forest pest.

Although our results are consistent, several caveats in our sampling need to be considered. First, we assumed that defoliation happened irrespective of the tree genotype in both study sites. However, as we studied pine plantations, usually with low genetic variation and intensive soil preparation, this bias may be not especially relevant because phenotypic and environmental variability is expected to be lower than in natural stands. Our results regarding tree chemical defenses can only be interpreted as a post-defoliation tree physiological status; therefore, we cannot speculate whether differences in chemical defenses influenced tree selection by the PPM. Second, chemical defensive differences between defoliated and non-defoliated trees could be the result of genotypic or phenotypic differences among individual trees that have conditioned the host selection by the PPM (Rivas-Ubach et al. 2017). In any case, several studies have reported a weak or unclear influence of tree volatiles and defensive chemistry on tree selection for oviposition by this insect (Hódar et al. 2002, Jactel et al. 2011), whereas oviposition is determined by stand structure and composition characteristics (Dulaurent et al. 2012, Hódar et al. 2002, Jactel et al. 2011, Régolini et al. 2014). For example, Régolini et al. (2014) hypothesized that PPM female could preferentially choose more apparent trees (i.e., trees located in stand edges) for oviposition. However, in our study, this was not likely the case, since we performed a paired selection of defoliated and non-defoliated trees in plantations in order to eliminate as much as possible

context differences between trees. Regarding our observations on the soil fungal community, our third caveat is that deposition of frass and dead needles resulting from larval activity could act as an organic fertilizer and indirectly affect the fungal community (Cigan et al. 2015, Nilsson and Wallander 2003). However, we discounted this possibility because defoliation did not result in a consistent increase in either nutrients or soil organic pools. Finally, our study was replicated in two sites differing in climatic conditions. Despite we find consistent above- and below-ground fungal responses to defoliation across sites, our data are limited to one wet and one dry site, which does not allow further conclusions to be drawn on how contrasting climatic conditions interact with insect defoliation. The influence of water availability on tree-insect interactions deserves further research.

Conclusions

Our results indicate that defoliation by PPM reduced tree radial growth and increased the production of some defense compounds in defoliated trees but did not affect sapwood concentrations of NSCs. Following these changes, we also observed an effect of defoliation over root-associated fungi, especially on ectomycorrhizal biomass, along with an increase of soil saprotrophs and pathogens. Interestingly, non-defoliated trees had distinct endophyte communities than defoliated trees, as we found specific indicator taxa associated to only non-defoliated trees. Our data do not allow us to clearly determine the actual cause of the reduction of ectomycorrhizal fungi in the soil, although it seems that the reduction in carbon availability for fungi in response to defoliation might reduce the soil ectomycorrhizal biomass. In any case, further research is needed to unravel the real mechanisms behind the observed patterns.

Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

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Conflict of interest

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

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Author contributions

C.C. conducted soil and needle sampling, community analysis and the statistical analysis of the data. J.J.C. conducted soil and tree sampling, the dendrochronology measurements, the tree C reserves quantification and the statistical analyses. R.Z. and L.S. conducted the tree samplings, measured the tree chemical defenses, determined soil and needle nutrients and performed statistical analyses. J.A.B., J.G.A. and J.O. conducted the soil and needle sampling for fungal studies, assisted with soil fungal measurements and contributed with the statistical analyses. J.O. also guided the main structure of the manuscript. All the authors conceived together the idea and the experiment. C.C. produced the first version of the manuscript, and all authors contributed equally to improve the writing with further revisions of the manuscript.

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