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Ectomycorrhizal fungal diversity decreases in Mediterranean pine forests adapted to recurrent fires

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Abstract

Fire is a major disturbance linked to the evolutionary history and climate of Mediterranean ecosystems, where the vegetation has evolved fire-adaptive traits (e.g., serotiny in pines). In Mediterranean forests, mutualistic feedbacks between trees and ectomycorrhizal (ECM) fungi, essential for ecosystem dynamics, might be shaped by recurrent fires. We tested how the structure and function of ECM fungal communities of *Pinus pinaster* and *Pinus halepensis* vary among populations subjected to high and low fire recurrence in Mediterranean ecosystems, and analysed the relative contribution of environmental (climate, soil properties) and tree-mediated (serotiny) factors. For both pines, local and regional ECM fungal diversity were lower in areas of high than low fire recurrence, although certain fungal species were favoured in the former. A general decline of ECM root-tip enzymatic activity for *P. pinaster* was associated with high fire recurrence, but not for *P. halepensis*. Fire recurrence and fire-related factors such as climate, soil properties or tree phenotype explained these results. In addition to the main influence of climate, the tree fire-adaptive trait serotiny recovered a great portion of the variation in structure and function of ECM fungal communities associated with fire recurrence. Edaphic conditions (especially pH, tightly linked to bedrock type) were an important driver shaping ECM fungal communities, but mainly at the local scale and probably independently of the fire recurrence. Our results show that ECM fungal community shifts are associated with fire recurrence in fire-prone dry Mediterranean forests, and reveal complex feedbacks among trees, mutualistic fungi and the surrounding environment in these ecosystems.

KEYWORDS

ectomycorrhizal communities, enzymatic activity, fire recurrence, Mediterranean pines, serotiny

1 | INTRODUCTION

Mediterranean climate is characterized by a marked seasonality with mild winters and hot dry summers, which gives rise to one of the most fire-prone ecosystems in the world (Pausas, 2004). The evolutionary history of these ecosystems is tightly linked to fire (Keeley, Bond, Bradstock, Pausas, & Rundel, 2011). Recurrent burning has markedly structured plant communities in this area (Verdú & Pausas, 2007), where many species have developed evolutionary mechanisms of resistance and resilience to fire (Pausas, 2015; Tapias, Climent, Pardos,

& a., & Gil, L., 2004). A good example of fire-adaptive trait is serotiny (i.e., the retention of mature seeds in closed cones for more than a year until dispersion and germination is activated by fire), which has evolved in some representative Mediterranean pine species (Budde et al., 2014; Hernández-Serrano, Verdú, González-Martínez, & Pausas, 2013) and other conifers in fire-dominated systems (Greene et al., 1999). Fire generates phenotypic divergence on serotiny, a heritable trait in Mediterranean pines, ultimately producing local adaptation (Hernández-Serrano et al., 2014). There is evidence that serotiny is related to differences in fire regime, increasing with the frequency of stand-replacing fires (Hernández-Serrano et al., 2013; Radeloff, Mladenoff, Guries, & Boyce, 2004). Changes in fire recurrence are closely linked to climate (Pausas, 2004), and predicted climate change scenarios make Mediterranean ecosystems especially vulnerable. Temperature rise and rainfall decrease are expected to increase drought risk and consequently forest wildfires, altering the structure and successional dynamics of Mediterranean forests (Lindner et al., 2010).

Ecosystem development is driven by interactions among climatic conditions, edaphic environment and biotic communities. Given the role of fungi in organic matter turnover and nutrient cycling, they are key players in the plant–soil–microbial feedbacks that determine ecosystem development after disturbances (Clemmensen et al., 2015). Specifically, mutualistic ectomycorrhizal (ECM) fungi, which are tightly linked to the host through specialized symbiotic structures, mediate the uptake and transfer of water and nutrients to trees (Smith & Read, 2008). They channel the photosynthetic carbon (C) fixed by trees into the below-ground system, influencing soil C storage and nutrient cycling (Clemmensen et al., 2013; Talbot, Allison, & Treseder, 2008). This symbiosis can provide up to the 80% of nutrients to trees and alleviate their hydric stress (van der Heijden, Martin, Selosse, & Sanders, 2015; Kivlin, Emery, & Rudgers, 2013), and therefore can be especially relevant in severe environments, such as the recurrent fire and drought conditions in Mediterranean ecosystems (Egerton-Warburton, Querejeta, & Allen, 2007; Prieto et al., 2016; Querejeta, Egerton-Warburton, & Allen, 2007).

Fire affects ECM fungal communities mainly through direct combustion, vegetation damages and altered soil properties (Buscardo et al., 2015; Hart, DeLuca, Newman, MacKenzie, & Boyle, 2005; Rincón & Pueyo, 2010), conditions that may favour fire-tolerant fungi (Glassman, Levine, Dirocco, Battles, & Bruns, 2016; Rincón, Santamaría-Pérez, Ocaña, & Verdú, 2014). Certain fungi can remain for long periods in the soil spore banks and be advantaged after fire, as shown in closed-cone pine forest populations with historical highly recurrent and intense fires (Baar, Horton, Kretzer, & Runs, 1999; Bruns et al., 2009; Glassman et al., 2015, 2016). Fire usually decreases root mycorrhizal colonization and fungal richness (Dove & Hart, 2017). More generally, fire drastically disrupts the species composition of the mycorrhizal communities, usually leading to their simplification with the dominance of pioneer fungi (Buscardo et al., 2015; Dove & Hart, 2017; Rincón et al., 2014; de Román & de Miguel, 2005; Torres & Honrubia, 1997). Additionally, because intraspecific plant genotypic and phenotypic variation affects the structure of their associated ECM fungi (Gehring & Whitham, 1991; van der Heijden et al., 2015; Pérez-Izquierdo et al., 2017, 2019) it is plausible that genetically fire-driven changes in trees, such as degree of serotiny, may co-affect these mutualistic communities. Nevertheless, fires recurrently affect large areas of Mediterranean ecosystems, yet the cumulative effects of recurrent wildfires on ECM fungal communities are less well known than those of single wildfires (Buscardo et al., 2015).

In the present study, we targeted forests of two representative Mediterranean tree species, Maritime pine (*Pinus pinaster* Ait.) and Aleppo pine (*Pinus halepen*sis Mill.), amply distributed in the Iberian Peninsula. While the Maritime pine usually grows in acid soils at 700–1,700 m altitude, the Aleppo pine grows in alkaline substrate and below 800 m (Ruíz, Álvarez-Uria, & Zavala, 2009). *P. halepensis* is typically distributed in warm and dry areas, even under extreme drought induced either by climate or by soil constituents (e.g., marls, gypsum, rocky slopes) (Ruíz et al., 2009). Both pine species have a life history related to fire adaptation (Tapias et al.., 2004) displaying great post-fire colonizing abilities (Barbéro, Loisel, & Quézel, 1998) and showing fire-adaptive traits such as serotiny (Pausas, 2015). The natural regeneration and dynamics of these pine forests critically relies on ECM fungal communities, because pine species are obligatory ECM (Nuñez, Horton, & Simberloff, 2009; Smith & Read, 2008). Recurrent fires, tightly linked to climate in these Mediterranean areas, might act as an environmental filter, culling plant and microbial species that are unable to tolerate conditions at a particular location and thus preventing their establishment or persistence (Kraft et al., 2015).

Based on all these premises, we expected that (a) due to the habitat filtering imposed by the fire recurrence and climate, the ECM fungal communities in high fire recurrence (hereafter HiFi) sites would be less diverse and more homogeneous than those in low fire recurrence (hereafter LoFi) sites, and that it would imply different functional outcomes. In our study area, the distinct historical fire recurrence has induced a sharp serotiny divergence in trees within the HiFi and LoFi populations over time (Hernández-Serrano et al., 2013). Serotiny has been shown to be a heritable trait associated with the genotype of trees (Budde et al., 2014; Castellanos, González-Martínez, & Pausas, 2015; Hernández-Serrano et al., 2013). Because different tree genotypes can influence their associated ECM fungi (Gehring & Whitham, 1991; Pérez-Izquierdo et al., 2017), we further hypothesize that, at the finer scale of individuals, (b) the serotiny degree of trees (i.e., genotypic fire-adaptation) will explain structural and functional divergences of ECM fungal communities.

To address these predictions, we characterized the structure of root-tip ECM fungal communities of natural *P. pinaster* and *P. halepensis* forests (Hernández-Serrano et al., 2013). For both pine forests, serotinous populations growing under a warm and dry Mediterranean climate subjected to high fire recurrence and nonserotinous populations growing under a subhumid climate where fires are rare (Verdú & Pausas, 2007) were surveyed (Figure S1). Additionally, we determined, on excised ECM root tips, potential fungal enzymatic traits related to C turnover and mobilization of nutrients. These are processes directly implicated in the exchange of resources that support most mycorrhizal symbioses and many essential ecosystem functions (Johnson, Martin, Cairney, & Anderson, 2012).

2 | MATERIAL AND METHODS

2.1 | Study sites and sampling

Surveys were conducted in natural forests of *Pinus pinaster* and of *Pinus halepensis* located in eastern Spain (Figure S1). We selected nine pine populations located in HiFi sites where crown-fires are historically frequent and most regeneration events are driven by fire (HiFi populations), while the other selected six populations were located in LoFi areas where most regeneration events are independent of fire because fire events are rare (LoFi populations) (Hernández-Serrano et al., 2013; Pausas, Bradstock, Keith, & Keeley, 2004). Seven populations corresponded to *P. pinaster* and eight to *P. halepensis*. In the study area, fire is tightly linked to Mediterranean climatic conditions (i.e., drought) (Pausas et al., 2004). Recent fire history information (Pausas, 2004; Pausas & Fernández-Muñoz, 2012) shows that more than 50% of the study area at HiFi conditions burned at least once during the period 1978–2001, while for LoFi conditions, the proportion was about 15% (Abdel Malak & Pausas, 2006). From a microevolutionary point of view, this distinct fire recurrence has induced a sharp serotiny divergence within these populations (Hernández-Serrano et al., 2013). More than 500 genes were differentially expressed across the two pine accessions from HiFi and LoFi populations (Pinosio et al., 2014) and three high-differentiation outlier single nucleotide polymorphisms (SNPs) were identified between HiFi and LoFi stands, suggesting fire-related selection at the regional scale (Budde et al., 2017). Thus, despite the lack of long-term fire statistics for the specific study sites, there is strong evidence that the fire interval is much shorter in HiFi areas than in LoFi areas. The degree of serotiny of these pine populations growing under distinct fire recurrence has been accurately characterized by Hernández-Serrano et al. (2013) (Table S1). Briefly, serotiny was estimated considering both the cone age and the proportion of serotinous cones, that is, the number of closed cones, those remaining closed after maturation, with respect to the total fully ripe (open and closed) cones (for details see Hernández-Serrano et al., 2013). The characteristics of pine populations related to productivity (diameter at breast height [DBH]), fire-adaptation traits (bark thickness and serotiny degree), as well as location and local environmental variables are described in Table S1. All the selected stands were mature pine populations, with DBH between 20.8 and 35.4 cm corresponding to trees of more than 40 years (Camarero, Olano, & Parras, 2010; Vieira et al., 2015).

In May 2013, five trees per population separated by more than 10 m were selected from a total of 15 populations (*n* = 75). Under each tree, the litter was removed and samples were obtained, ~1 m from the trunk, by excavating 10 × 10 × 20-cm soil holes at the four orientations (north, south, east and west). The four samples per tree were pooled into a combined sample and kept at 4°C in plastic bags until processing. Once in the laboratory, roots were separated from soil, coarse roots were discarded (diammeter> 2 mm), and remaining roots were gently washed with tap water over 2- and 0.5 mm sieves for collecting root tips. All ECM root tips per each sample were carefully selected (Rincón et al., 2014), cleaned and sorted per sample under a stereomicroscope for further enzymatic and molecular analyses. Remaining soil was air dried and sieved (2 mm) for analysis.

The gravimetric soil moisture of soil samples was determined by drying at 105°C for 48 hr. Air-dried soils were measured for pH (2 g of soil in 10 ml of H_2O, 1:5, w/v), electrical conductivity (EC) (1:5, w/v in H_2O), organic matter (OM) (Walkley & Black, 1934) and total N (Kjeldahl method). Extractable phosphorus (P) was determined by the Bray and Kurtz (1945) method, after extraction in an ammonium fluoride and chloride acid solution. Extractable potassium (K) was determined after nitric acid digestion according to Isaac and Kerber (1971). Both P and K extracts were measured by inductively coupled plasma spectrometry (Optima 4300DV, Perkin-Elmer).

2.2 | Enzymatic tests

Fungal community functioning was evaluated on excised ECM root tips by measuring activities of eight hydrolytic and oxidative exoenzymes secreted by fungi. Seven enzymatic tests were based on fluorogenic substrate release, methylumbelliferone (MU) (e.g., β-glucosidase [EC 3.2.1.3 at ExPasy-Enzyme database]) and cellobiohydrolase (EC 3.2.1.91) that degrade cellulose, β-xylosidase (EC 3.2.1.37) and β-glucuronidase (EC 3.2.1.31) implied in the degradation of hemicellulose, acid phosphatase (EC 3.1.3.2) involved in the mobilization of phosphorus, N-acetylglucosaminidase or chitinase (EC 3.2.1.14) which hydrolyses chitin, or methylcoumarine (AMC) for L-leucineaminopeptidase (3.4.11.1) related to the mobilization of nitrogen from peptidic substrates. Laccase (1.10.3.2) activity was determined by a photometric assay based on ABTS substrate (2,2′-azino-bis (3-ethylbenzo-thiazolin-6-sulfonic acid)). This enzyme is related to the degradation of recalcitrant compounds such as lignin. Enzymatic activities were determined following the protocol described by Courty, Pritsch, Schloter, Hartmann, and Garbaye (2005), with modifications. A total of 280 ECM-tips were randomly collected per sample and separated in subsets of seven ECM-tips with five replicates per each enzymatic test. Each replicate thus consisted of a tube with seven pooled ECM-tips that were incubated in buffer during the corresponding time for each enzyme (Courty et al., 2005), after which 100 μl of the respective enzymatic reaction mix was added to 100 μl of stopping buffer in 96-well microplates. Enzymatic activities were measured in a Victor microplate reader (Perkin-Elmer Life Sciences), with 355/460 nm excitation/emission wavelengths for the fluorogenic assays and 415 nm for laccase. After reading, the ECM-tips of each replicate were scanned and their area was calculated with the software IMAGEJ 1.49. Enzymatic activities were expressed in pmol/min mm^{-2}.

2.3 | Molecular analyses

For each measured enzyme, the ECM-tips were pooled (7 ECM-tip subsets × 5 replicates = 35), a small amount of polyvinylpolypyrrolidone (PVPP) was added and the DNA was extracted with the Invisorb DNA Plant HTS 96 Kit/C kit (Invitek), making a total of 600 DNA extractions (75 tree samples × 8 enzymes) corresponding to 280 root tips per tree.

The internal transcribed spacer region ITS-1 of the nuclear ribosomal DNA was amplified with the primer pair ITS1F–ITS2 (Gardes & Bruns, 1993) adapted for Illumina-MiSeq. Each sample was amplified in three independent 20-µl PCRs (polymerase chain reactions), each containing 2 µl of 10 × polymerase buffer, 2.4 µl of 25 mm $MgCl_2$, 1.12 µl of 10 mg/ml bovine serum albumin, 0.4 µl of 10 mm Nucleotide Mix, 0.4 µl of 10 mm forward/reverse primers and 0.2 µl of AmpliTaqGold polymerase (5 U/ml) (Applied Biosystems). Negative controls without DNA were included in all runs to detect possible contaminations. The PCR conditions were as follows: 3 min at 94°C, 30 cycles of 1 min at 94°C, 30 s at 53°C and 45 s at 72°C, with a final step of 10 min at 72°C. Independent reactions were combined per sample, and each PCR product was purified (UltraClean PCR clean-up kit of MoBio), quantified (PicoGreen, Life Technologies), and finally pooled in an equimolar library containing 75 samples. Sequencing was carried out on an Illumina MiSeq sequencer (2 × 300-bp paired-end reads) in an external service (Parque Científico de Madrid).

2.4 | Bioinformatic analyses

Sequences were demultiplexed according to their tags, filtered and trimmed using the *fastq_filter* command and *fastq_truncqual* option of USEARCH version 7.0.1001 (Edgar, 2013) for eliminating quality scores ≤ 10. We used FLASH to merge reads with a minimum overlap of 110 bp, resulting in 58.3% of retained sequences (4,205,677 out of the initial set of 7,215,915 sequences). Then, sequences were dereplicated with the *derep_fulllength* USEARCH command. Dereplicated sequences were sorted by decreasing abundance, and singletons were discarded with the *sortbysize* USEARCH command. Finally, 4,116,377 sequences (57%) were retained. Operational taxonomic units (OTUs) were generated from abundance-sorted sequences using the *cluster_otus* USEARCH command with a 97% similarity threshold. Extracted ITS sequences were then mapped against the OTU representative sequences using the *usearch_global* USEARCH command. Taxonomic assignation of representative sequences for each OTU was done according to the 7.1 UNITE database with accession date 20 November 2016 (Kõljalg et al., 2013). Fungal taxonomic assignment served to identify those OTUs closely related to recognized ECM taxa (Nguyen et al., 2016; Tedersoo et al., 2014; Tedersoo & Smith, 2013). In total, 86% of the inferred sequences corresponded to ECM fungi and these were used for all subsequent analyses.

2.5 | Statistical analyses

The effect of fire recurrence (HiFi versus LoFi) on the structure (i.e., α- and β-diversity) and functioning (i.e., enzymatic activities) of ECM fungal communities (hypothesis 1) was separately evaluated by pine forest. The dissimilarity degree of total and per-phyla ECM fungal communities across the different pine populations (i.e., β-diversity) was evaluated with the functions ANOVA and permutest on the multivariate dispersion object calculated by the betadisper function of the VEGAN package (Anderson, Ellingsen, & McArdle, 2006; Oksanen et al., 2015), and visualized by principal component analyses (PCAs). Beta-diversity was calculated on the Bray–Curtis abundance matrix of OTUs previously normalized by variance stabilization according to McMurdie and Holmes (2014) by using the DESEQ package (Anders & Huber, 2012).

Alpha-diversity was analysed by fitting an analysis of variance to a linear model (LM) ($p < .05$) with the number of fungal OTUs (i.e., richness) as the response variable. We used the Akaike information criterion to choose the best fitting model. In these models, the square root of the total number of sequences obtained per sample was included as a covariate to account for differences in sequencing depth. Models at lower taxonomic levels were also performed (i.e., phyla and families). The *p*-value in these models was corrected by the Bonferroni–Holm method to account for multiple comparisons. In all cases, before modelling, Shapiro and Levene tests were performed to test the normality and homocedasticity of the data, and variables were log or square root transformed when needed. First, we modelled the effect of the variable pine forest on ECM fungal α-diversity, using pine forest as the fixed factor and site nested within pine forest. Subsequent LM analyses were separately performed for each pine species. In these models, fire recurrence was introduced as a fixed factor and site (i.e., pine population) nested within fire recurrence ($n = 35$ *P. pinaster* trees and $n = 40$ *P. halepensis* trees). The same LM syntaxes were used to test the effect of fire recurrence on the different root-tip enzymatic activities. Correlations between enzymatic activities and ECM fungal community assemblages of the pine populations were analysed by Mantel tests with the ADE4 R package.

To identify representative fungal OTUs of each fire recurrence per pine species, indicator species analysis (with OTUs> 100 reads to avoid spurious relations) was carried out ($p < .05$) with the function multipatt of the INDICSPECIES R package (Cáceres, Legendre, & He, 2013).

Climatic and edaphic characteristics are intimately associated with fire recurrence in Mediterranean ecosystems (Pausas et al., 2004). So, to separate the edaphic and climatic effects from that of fire recurrence, we performed canonical correspondence analysis (CCA) on Hellinger-transformed relative fungal abundance data and redundancy analysis (RDA) with the Euclidean distance matrix of enzymatic activities both with forward selection of explanatory variables. We used different analyses due to their different data assumptions: RDA assumes a linear relationship between predictors and data (i.e., the case for enzymatic activities), while CCA assumes a unimodal response curve (i.e., the case for ECM fungal community data). In these models, the variable fire recurrence and the edaphic and climatic indexes were included. Previously, to reduce the dimensionality of environmental data, PCAs were independently run for edaphic variables (pH, OM, EC, N, P, K) and climatic variables (mean annual temperature, mean annual precipitation and altitude), and the first two axes, explaining the maximum amount of variance, of respective PCAs were used as edaphic or climatic indexes (Figure S2).

To test hypothesis 2, the effect of tree serotiny degree (i.e., genotypic fire-adaptation) on ECM fungal community, two additional models with the factor serotiny and the edaphic and climatic indexes were included. As before, we performed a CCA model on Hellinger-transformed relative fungal abundance data and an RDA model with the Euclidean distance matrix of enzymatic activities both with forward selection of explanatory variables.

Because the distribution of the two pine species considered in our study is closely linked to bedrock type—i.e., *P. pinaster* preferentially settled on siliceous soils, while *P. halepensis* included both siliceous and calcareous soils (Hernández-Serrano et al., 2013)—we made additional analyses for testing possible confounding effects of pine species and bedrock type (both factors included in the variable pine forest). For this, the pine species effect on ECM fungal community structure was checked by CCA and on enzymatic activities by RDA, only with data of Sinarcas, the unique location where populations of both pine species grow under a common bedrock environment (Table S1). No significant effects of pine species identity on the ECM fungal community structure (CCA: n = 218; explained variation% = 12.4; p = .204) or enzymatic profile (RDA: n = 8; explained variation%=44.4; p = .094) in the location Sinarcas were observed.

Ordinations were done with the software CANOCO version 5.0 (Biometris Plant Research International), and the remaining analyses with the R software version 3.5.2 (R Core Team, 2014).

3 | RESULTS

3.1 | Sequencing yields and taxonomic identification of fungi

A total of 501 ECM fungal OTUs corresponding to 3,182,548 sequences were identified. Almost half of the OTUs were found in both pine species and ~26% exclusively in *Pinus pinaster* and the same percentage exclusively in *Pinus halepensis* (Figure S3a). LoFi and HiFi populations shared 54.8% of OTUs, while ~21%–23% were only found either in LoFi or in HiFi (Figure S3a). Most OTUs belonged to Basidiomycota (89.4%), followed by Ascomycota (10.2%) and Zygomycota (0.4%) (Figure S3b; Table S2). The taxonomic classification of OTUs allowed us to identify 12 fungal orders, 30 families and 47 genera, most of them represented across all treatments (Table S2).

The majority of the 20 most abundant fungi and of the indicator species found in root tips of *P. pinaster* belonged to *Tomentellaceae*, *Inocybaceae*, *Russulaceae*, and *Rhizopogonaceae* and, in the case of *P. halepensis*, to Pezizales, *Thelephoraceae* and *Sebacinaceae* (Table S3; Figure S4). The indicator species analysis revealed 54 fungal OTUs preferentially associated with *P. pinaster* forests and 37 with *P. halepensis* forests (Table S3). In total, 31 OTUs belonging to 14 different genera were indicators of LoFi populations of *P. pinaster* and 31 OTUs belonging to 13 different genera were indicators of LoFi populations of *P. halepensis*. In contrast, 11 indicators from five different genera and six indicators from five different genera were associated with HiFi populations of *P. pinaster* and *P. halepensis* respectively (Table S3).

3.2 | Root-tip ECM fungal diversity

Total fungal α-diversity varied marginally between pine forests ($F_{1,74}$ = 3.60; p = .062). At the phylum level, significantly fewer Basidiomycetes ($F_{1,74}$ = 6.12; p = .016) and marginally more Ascomycetes ($F_{1,74}$ = 3.88; p = .053) were found in *P. halepensis* forest compared with *P. pinaster* forest. The root tips of *P. pinaster* were enriched in *Amanitaceae*, *Atheliaceae*, *Cantharellaceae*, *Clavariaceae*, *Cortinariaceae*, *Gloniaceae*, *Rhizopogonaceae* and *Russulaceae* species, while *P. halepensis* root tips were dominated by species of the families *Pezizaceae*, *Pyronemataceae* and *Sebacinaceae* (Table 1). At the regional scale, root-tip ECM fungal β-diversity of both pine forests was similar ($F_{1,74}$ = 1.34, p = .265), although Basidiomycetes were more dissimilar in *P. pinaster* than in *P. halepensis* forests ($F_{1,74}$ = 4.44, p = .033), and the opposite pattern was observed for Ascomycetes ($F_{1,74}$ = 11.62, p = .001).

The ECM fungal α-diversity was significantly lower in HiFi populations compared with LoFi populations in both pine forests (Figure 1a), and this difference was consistent within the two fungal phyla (i.e., Ascomycetes and Basidiomycetes; Figure 1b,c). Although the α-diversity of ECM fungal families generally decreased in HiFi populations of both pine forests (Table 1), some taxa were more diverse in HiFi (e.g. Amanitaceae, Cantharellaceae and marginally Rhizopogonaceae for *P. pinaster*; Table 1). At the regional scale, a consistent reduction of ECM fungal β-diversity was observed in HiFi populations of both pine forests (i.e., more homogeneous ECM communities in HiFi than LoFi) (Figure 2a). Likewise, Basidiomycetes had lower β-diversity in HiFi than LoFi populations, while no difference was observed for Ascomycetes (Figure 2b,c).

3.3 | Potential enzymatic activity of ECM root tips

The enzymatic activity of the ECM root tips varied between pine forests (Figure 3). In LoFi populations, the enzymatic activity of ECM root tips was very similar for both pines, except in the case of cellobiohydrolase and phosphatase which were respectively higher and lower in *P. halepensis* compared with *P. pinaster* (Figure 3). By contrast, in HiFi populations, the activity of most of the C-degrading enzymes (i.e., cellobiohydrolase, β-xylosidase and β-glucuronidase), and of those mobilizing nitrogen (i.e., chitinase and

TABLE 1 Alpha-diversity of representative ectomycorrhizal (ECM) fungal families analysed by linear models ($p < .05$) [Colour table can be viewed at wileyonlinelibrary.com]

	Pine Forest			*P. pinaster*		*P. halepensis*	
	Ppi	*Pha*	$F_{1,74}$ (p_{adj})	LoFi	HiFi	LoFi	HiFi
Amanitaceae^{B}	=	=	13.02 (0.002)	**0.1 ± 0.1 a**	**0.9 ± 0.2 b**	0.1 ± 0.1	0.2 ± 0.1
Atheliaceae^{B}	=	=	7.6 (0.064)	**9.7 ± 0.6 b**	**6.7 ± 0.6 a**	6.3 ± 0.5	5.9 ± 0.4
Bankeraceae^{B}	=	=	2.3 (0.870)	1.8 ± 0.4	1.3 ± 0.3	1.7 ± 0.5	0.9 ± 0.2
Cantharellaceae^{B}	↑		**43.1 (< 0.001)**	**1.0 ± 0.2 a**	**2.8 ± 0.4 b**	0.7 ± 0.2	0.5 ± 0.2
Clavariaceae^{B}	↑		**37.6 (< 0.001)**	0.8 ± 0.2	1.2 ± 0.4	0.3 ± 0.2	0.1 ± 0.1
Clavulinaceae^{B}	=	=	2.9 (0.662)	2.9 ± 0.3	1.7 ± 0.3	2.7 ± 0.5	1.3 ± 0.3
Cortinariaceae^{B}	↑		**18.9 (< 0.001)**	**2.2 ± 0.7 b**	**0.4 ± 0.2 a**	0.6 ± 0.4	0.2 ± 0.1
Gloniaceae^{A}	=	=	8.7 (0.04)	2.5 ± 0.3	1.2 ± 0.2	1.7 ± 0.4	0.9 ± 0.3
Hydnaceae^{B}	=	=	0.8 (1.00)	0.3 ± 0.1	0.2 ± 0.1	0.6 ± 0.2	0.2 ± 0.1
Inocybeaceae^{B}	=	=	1.6 (1.00)	5.5 ± 0.8	3.0 ± 0.5	3.1 ± 0.3	2.8 ± 0.3
Pezizaceae^{A}		↑	**26.2 (< 0.001)**	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.2	1.1 ± 0.2
Pyronemataceae^{A}		↑	**33.6 (< 0.001)**	0.6 ± 0.2	0.9 ± 0.2	**2.9 ± 0.3 b**	**1.6 ± 0.3 a**
Rhizopogonaceae^{B}	↑		**27.6 (< 0.001)**	0.9 ± 0.2	1.8 ± 0.2	0.8 ± 0.2	0.4 ± 0.1
Russulaceae^{B}	↑		**15.0 (0.003)**	7.3 ± 0.6	5.5 ± 0.7	4.3 ± 0.8	4.3 ± 0.5
Sebacinaceae^{B}		↑	**33.9 (< 0.001)**	5.7 ± 1.1	4.5 ± 0.4	8.7 ± 0.8	9.0 ± 0.8
Suillaceae^{B}	=	=	0.02 (0.00)	1.6 ± 0.3	1.2 ± 0.2	1.6 ± 0.2	1.2 ± 0.2
Telephoraceae^{B}	=	=	0.01 (0.00)	11.9 ± 1.0	11.0 ± 1.0	14.1 ± 1.8	10.4 ± 0.8
Tuberaceae^{A}	=	=	0.03 (0.00)	1.4 ± 0.3	1.6 ± 0.3	1.6 ± 0.3	1.4 ± 0.2

Note: Main effects of pine species (Ppi = *Pinus pinaster* and Pha = *Pinus halepensis*) (left) and fire recurrence (LoFi = low fire recurrence and HiFi = high fire recurrence) (right) are shown. Site was nested within the fixed factor fire recurrence in the models. Data represent means ± *SE*. Arrows indicate significantly higher α-diversity, and "=" indicates no variation between pine species according to the model (*F* values, *df*, *p*-value corrected by the Bonferroni–Holm method [*p*-adj]). In the separate analyses on the right, for each pine forest, different letters denote significant differences between fire recurrences ($p < .05$) (in bold). A = Ascomycetes (orange); B = Basidiomycetes (green).

L-leucineaminopeptidase) was higher for *P. halepensis* than for *P. pinaster*, with the exception of laccase which was lower (Figure 3).

The ECM fungal species assemblage of *P. pinaster* root tips correlated significantly with their enzymatic profile (Mantel $r = 0.14$; $p = .003$). The HiFi populations of *P. pinaster* had higher root-tip laccase activity and lower hemicellulose- degrading activity (i.e., β-xylosidase and β-glucuronidase), and of enzymes mobilizing nitrogen (i.e., chitinase and L-leucineaminopeptidase) and phosphorus (i.e., acid phosphatase) (Figure 3). No significant relationship between fungal community structure and enzymatic activity was observed for *P. halepensis* (Mantel $r = 0.03$; $p = .287$), although laccase activity increased significantly in HiFi populations (Figure 3).

3.4 | Main drivers of the structure and function of ECM fungal communities

Climate, in particular temperature (Climate 1, first axis from PCA in Figure S2), together with fire recurrence were major factors determining ECM fungal communities of Mediterranean pine forests (Table S4A; Figure 4a). Additionally, the tree trait serotiny (selected by recurrent fires) did affect the structure of root-tip ECM fungal communities (Table S4B; Figure 4a). Higher levels of serotiny correlated significantly with the ECM fungal community structure of HiFi populations in both *P. pinaster* and *P. halepensis* forests (Table S4B; Figure 4a). By contrast, the edaphic effects were probably related to differences among plots inside LoFi or HiFi populations (i.e., the strength and direction of edaphic vectors along plots displayed in Figure 4a).

Regarding ECM fungal functionality (i.e., enzymatic activities), fire recurrence together with climatic variables explained root-tip enzymatic activity in *P. pinaster* forest (Table S4A; Figure 4b). Moreover, the degree of serotiny of *P. pinaster* populations correlated significantly with the ECM root-tip enzymatic profile in HiFi populations, while soil characteristics mainly drove variations in enzymes among sites inside LoFi and HiFi populations (Table S4B; Figure 4b). For *P. halepensis*, root-tip enzymatic activity was essentially driven by the surrounding edaphic environment (Table S4; Figure 4b), mainly by high pH and low phosphorus availability (see PC2 in Figure S2a).

4 | DISCUSSION

Mutualistic feedbacks between trees and ECM fungi that are essential for ecosystem dynamics might be shaped by historical recurrent fires, which are tightly linked to climate in Mediterranean ecosystems. Our study shows that structural and functional shifts in root-tip ECM fungal communities are associated with distinct fire

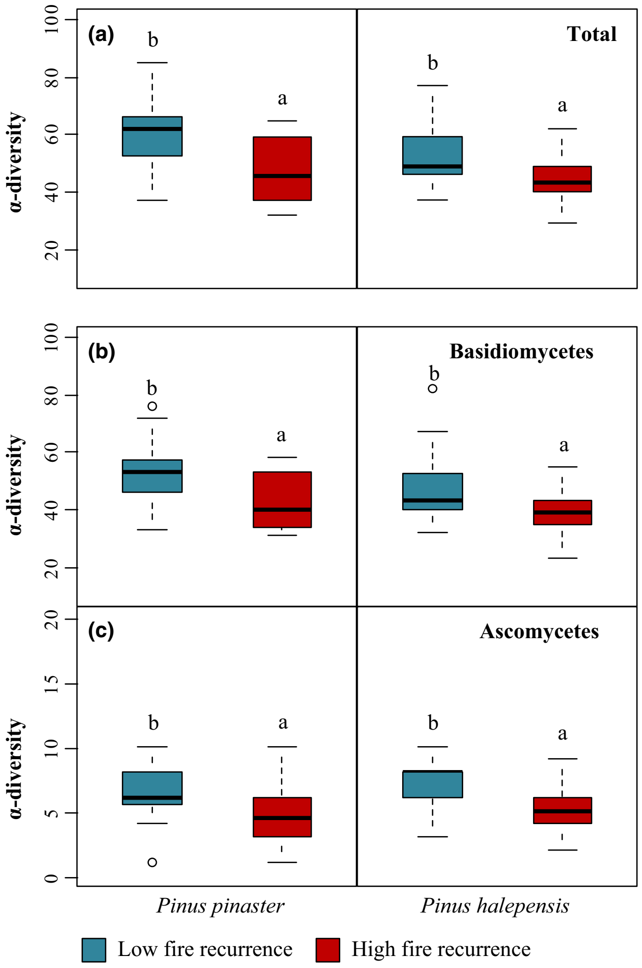

FIGURE 1 Alpha-diversity of (a) total, (b) Basidiomycetes and (c) Ascomycetes ectomycorrhizal fungal communities associated with root tips of low (blue) or high (red) fire recurrence populations of *Pinus pinaster* and *Pinus halepensis*, analysed by linear models ($p < .05$). Boxes represent the interquartile range (IQR) between first and third quartiles and the horizontal line inside is the median. Whiskers denote the lowest and highest values within 1.5 × IQR from the first and third quartiles, respectively. Within each graph, different letters denote significant differences among fire recurrences [Colour figure can be viewed at wileyonlinelibrary.com]

recurrences in fire-prone Mediterranean forests. *Pinus pinaster* and *Pinus halepensis* harbour different ECM fungal communities, which are in turn highly influenced by the edaphic environment and climate. According to our first hypothesis, lower ECM fungal diversity is found in HiFi populations compared with LoFi populations, which leads to a reduced enzymatic activity for *P. pinaster* (but not for *P. halepensis*). Confirming our second hypothesis, this effect is further evidenced indirectly through potential fire effects on tree individuals because the serotiny degree of trees (i.e., genotypic fire-adaptation) does significantly affect the structure (for both *P. pinaster* and *P. halepensis*) and functioning (in the case of *P. pinaster*) of ECM fungal communities. Here, we also disentangle the relative contribution of fire, climate, soil properties and the tree trait serotiny, structuring ECM fungal communities, although it cannot be ruled out that other underlying factors also operate.

4.1 | ECM fungal communities in *P. pinaster* and *P. halepensis* forests

Tree species had a marginal effect on ECM fungal communities probably blurred by the strong effect of the local environmental conditions. The degree of tree host specificity for ECM fungi seems to be correlated with the phylogenetic distances between hosts (Tedersoo, Mett, Ishida, & Bahram, 2013). However, it has been shown that the genus *Pinus* associates with specialized ECM fungi, in particular within the genera *Suillus* and *Rhizopogon* (Smith & Read, 2008) and that even the *Pinus* genotype plays an essential role in structuring ECM fungal communities (Gehring, Mueller, Haskins, Rubow, & Whitham, 2014; Pérez-Izquierdo et al., 2017, 2019). Given the importance of pH shaping fungal communities (Coince et al., 2014; Glassman, Wang, & Bruns, 2017; Rincón et al., 2015; Tedersoo et al., 2014), these divergent observations could be interpreted as context-dependent—i.e., in our study, soil pH (ranging from 5.1 to 8) was higher, even in Sinarcas where both pine species grew under a similar local environment, than in the mentioned studies (average pH of 5). The strong environmental filter imposed by the influence of bedrock, mainly siliceous for *P. pinaster* and calcareous for *P. halepensis* (Ojeda, Pausas, & Verdú, 2010), was reflected in more diverse and more heterogeneous Ascomycetes species together with less diverse and more homogeneous Basidiomycetes in the case of *P. halepensis*, whereas the opposite pattern was observed for *P. pinaster*. We observed a preferential association of *P. halepensis* with fungal species of the genus *Sebacina* and the order Pezizales, as well as single species often related to alkaline soils such as *Suillus collinitus* and *Lactarius sanguifluus*. So far, no tree host specificity has been observed among *Sebacina* species (Ray & Craven, 2016; Selosse, Bauer, & Moyersoen, 2002). Given the reported positive effects of sebacinous fungi against stresses such as herbivory, salinity or drought (Barazani & Baldwin, 2013; Ray & Craven, 2016; Zarea, Miransari, & Karimi, 2014), they could play a key role in the resistance of *P. halepensis* to limited nutrient availability (i.e., related to high soil pH) and the restrictive conditions imposed by the Mediterranean climate. Similarly, ascomycetous ECM Pezizales, which tend to be favoured in alkaline soils, have traits such as their melanin production capacity that make them perfect symbionts under stressful conditions including following fire (Koide, Fernandez, & Malcolm, 2014; Lamit et al., 2014; Rincón & Pueyo, 2010; Rincón et al., 2014; Tedersoo, Hansen, Perry, & Kjøller, 2006; Torres & Honrubia, 1997). On the other hand, species from the genera *Rhizopogon*, *Craterellus*, *Cenoccocum*, *Russula* and *Lactarius* were enriched under *P. pinaster* (less basic pH). Although ECM fungi tolerate a wide range of pH (Rousk et al., 2010), most of these fungi grow better under acidophilic conditions (Yamanaka, 2003).

4.2 | Structural and functional shifts of root-tip ECM fungal communities in LoFi and HiFi populations

As we first hypothesized, for both pine species, the local and regional diversity of root-tip ECM fungi significantly declined in populations

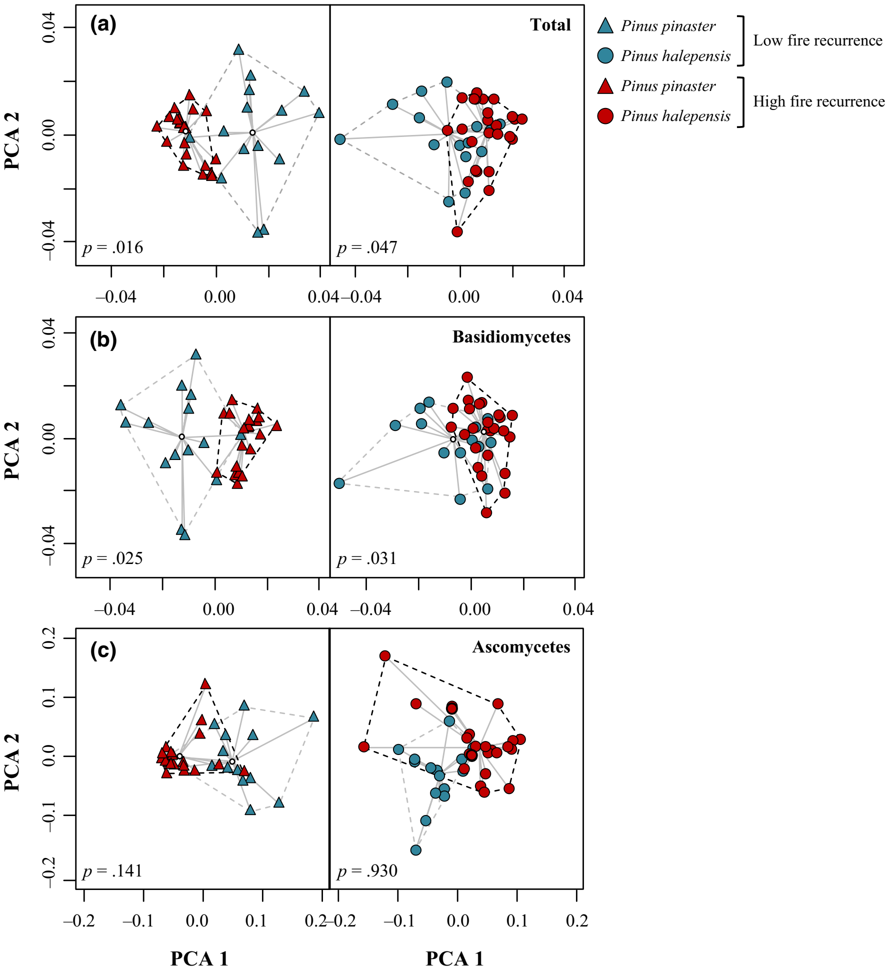

FIGURE 2 Beta-diversity of (a) total, (b) Basidiomycetes and (c) Ascomycetes ectomycorrhizal fungal communities associated with root tips of low (blue) or high (red) fire recurrence populations of *Pinus pinaster* (triangles) and *Pinus halepensis* (circles). The centroids within each group are represented by small white dots. Grey solid lines give the relative position of the point with respect to the respective centroid. Dotted lines reflect the area occupied by all plots of a given treatment, which is inversely proportional to the similarity of their ECM fungal communities [Colour figure can be viewed at wileyonlinelibrary.com]

where fires are historically frequent and associated with a warm and dry climate, and this implied functional outcomes (e.g., laccase activity of ECM root tips increased in HiFi populations of both pine forests). Many fire studies have ascribed decreases in fungal diversity to the reduction of heat-resistant propagules, elimination of rare species from the spore bank, the scarcity of suitable hosts, the direct burning of mycelium and roots, and/or the disturbed soil properties (Glassman et al., 2016; Holden, Gutierrez, & Treseder, 2013; Rincón & Pueyo, 2010). These processes do have a great importance on ECM fungal communities in the short term after disturbance, leading to other processes such as dispersion through time (Dooley & Treseder, 2011; Kipfer, Moser, Egli, Wohlgemuth, & Ghazoul, 2011; Rincón et al., 2014; Sun et al., 2015) similar to that reported in succession studies, where fungal richness increases with forest age (Kyaschenko, Clemmensen, Hagenbo, Karltun, & Lindahl, 2017; Twieg, Durall, & Simard, 2007). In our study, it seems that ECM fungal diversity has been restored since the last fire (at least more than 40 years ago), because typical late-stage species such as *Amanita* or *Lactarius* (Cairney & Chambers, 2013; Taylor & Bruns, 1999) were indicators in HiFi areas. Different climatic pressure and historical fire recurrences might have driven plant–soil feedbacks to different directions and selected species of ECM fungi that respond more efficiently to the new environmental conditions (Duhamel et al., 2019). Note that we compared pine populations with different fire-related traits (indirect fire recurrence effects), and that direct fire recurrence effects on ECM fungal communities are difficult to separate from other aspects (e.g., geographical, evolutionary) influencing tree–soil feedbacks, which could somehow limit interpretation of the results.

The ECM species composition diverged between LoFi and HiFi areas and, although fungal diversity decreased with HiFi, some ECM fungal species were overrepresented in HiFi populations, such as species within the families *Amanitaceae*, *Cantharellaceae* and *Rhizopogonaceae* for *P. pinaster*, or *Terfezia* within the family *Pezizaceae* in *P. halepensis*. Different authors have proposed that in ecosystems subjected to frequent fires, the positive response of microorganisms would indicate a selection of the fire-tolerant ones over time (Buscardo et al., 2015; Dooley & Treseder, 2011; Rincón et al., 2014). Fire recurrence in Mediterranean forests is intimately linked to temperature and drought (Pausas, 2004), so as expected, climate was, aside from fire recurrence, a strong determinant of fungal community shifts. All these results, together with the observed decline in ECM fungal β-diversity, seem to indicate that the habitat filtering was probably the dominant ecological process assembling root-tip ECM fungal communities under HiFi, while without the fire pressure (i.e., LoFi) and milder climate conditions, competition among fungi could prevail, similar to that described across biological groups (Pérez-Valera, Verdú, Navarro-Cano, & Goberna, 2018; Verdú & Pausas, 2007). The diversity of

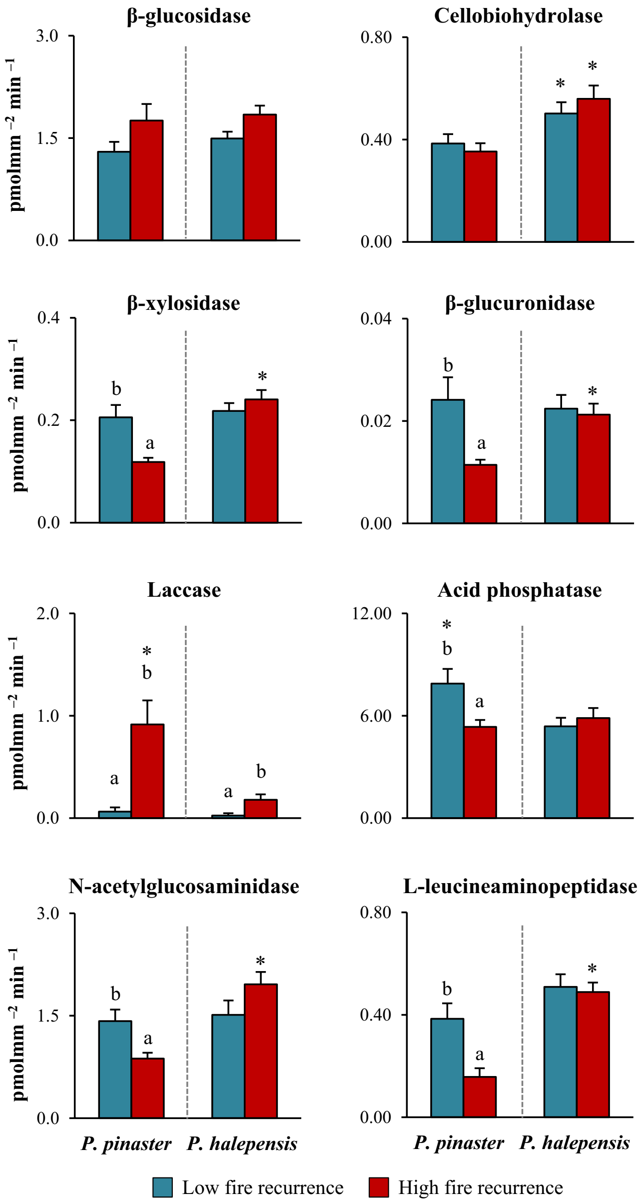

FIGURE 3 Enzymatic activity of ectomycorrhizal root tips (means ± *SE*) of low (LoFi, blue) or high (HiFi, red) fire recurrence populations of *Pinus pinaster* and *Pinus halepensis*, analysed by linear models ($p < .05$). For each pine species, different letters denote significant differences between fire recurrence levels, while for each LoFi/HiFi treatment, asterisks denote significant differences between pine forest levels [Colour figure can be viewed at wileyonlinelibrary.com]

plant and microbial communities is regulated by sequentially operating assembly rules: abiotic filtering is an omnipresent structuring force, and biological interactions (e.g., competition) further fine-tune the community assemblages (Pérez-Valera et al., 2018; Verdú & Pausas, 2007).

Fire recurrence and climate can filter directly different biotic organisms (i.e., trees and ECM fungi), but our results did provide significant clues about indirect plant-mediated effects of high fire recurrence on ECM fungal communities. We observed that serotiny, previously demonstrated as a tree heritable trait shaped by recurrent fires (Budde et al., 2014; Castellanos et al., 2015; Hernández-Serrano et al., 2014), significantly explained the variation of ECM fungal communities, aside from the accounted climatic effects for both pine species. This could be attributed to climatic constraints in the channel of C from the tree towards the ECM fungi, although, on the other hand, serotiny is a trait that requires resources (water and C) to maintain a large amount of seeds alive in the canopy with a cost for the plant (Cramer & Midgley, 2009; Harris & Pannell, 2010). Another possible explanation is that the ability of the different tree genotypes/phenotypes to preferentially allocate photosynthates to the more beneficial ECM fungi, or to those with low C demands and/or high abilities to cope with hydric/nutrient stress can be the prevailing mechanism explaining such plant-mediated effects (Bever, Richardson, Lawrence, Holmes, & Watson, 2009; Gehring et al., 2014). This was to some extent supported by our results, at least in *P. pinaster*, for which a direct effect of tree phenotype (serotiny) on enzymatic responses of ECM fungi but no effect of climatic variables was observed. We have previously shown that particular *P. pinaster* genotypes can impact enzymatic activities through changes in the composition of the associated microbial communities (Pérez-Izquierdo et al., 2017, 2019). Additionally, it cannot be ruled out that fire recurrence negatively affects other tree traits such as root development and/or architecture constraining the space for ECM fungal colonization, which would make mechanisms such as priority effects particularly important (Kennedy, Peay, & Bruns, 2009; Peay, Belisle, & Fukami, 2012). Regardless, further experimentation would be needed to shed light on these assumptions.

Changes in ECM fungal community structure were translated into differential functional responses in the two pine species, with lower enzymatic activities in HiFi populations in the case of *P. pinaster*, but not for *P. halepensis*. This suggests functional redundancy between ECM fungal communities associated with *P. halepensis* in LoFi and HiFi sites, because diversity and assemblage shifts were not translated into functional changes (Jones et al., 2010). These results might indicate a high capacity of *P. halepensis* forests to recover their functionality among recurrent fires in warmer areas probably by shifting plant–microbial feedbacks to readjust to the environment (Clemmensen et al., 2015; Johnstone et al., 2010). Edaphic conditions (mainly pH and P) were the main factors explaining differences in enzymatic profiles among populations of both pine species. Similarly, Brunel, Silva, and Gros (2020) observed that edaphic parameters, and not climate and vegetation, significantly explained microbial functioning patterns in Mediterranean soils. Among their multiple functions, an outstanding feature of ECM fungi is their ability to mobilize nutrients from the soil (Smith & Read, 2008). Because of their direct access to C from the host, they are able to invest energy (i.e., enzymatic production) to mine for N and/or P from hardly accessible sources (Fernandez, See, & Kennedy, 2020; Lindahl & Tunlid, 2015), as could be the case for alkaline soils in our study together with recalcitrant pine litter. Our findings are in line with previous studies indicating that the availability of resources in surrounding soil together with tree host genetics strongly regulate ECM enzymatic production (Aponte, García, Marañón, & Gardes, 2010;

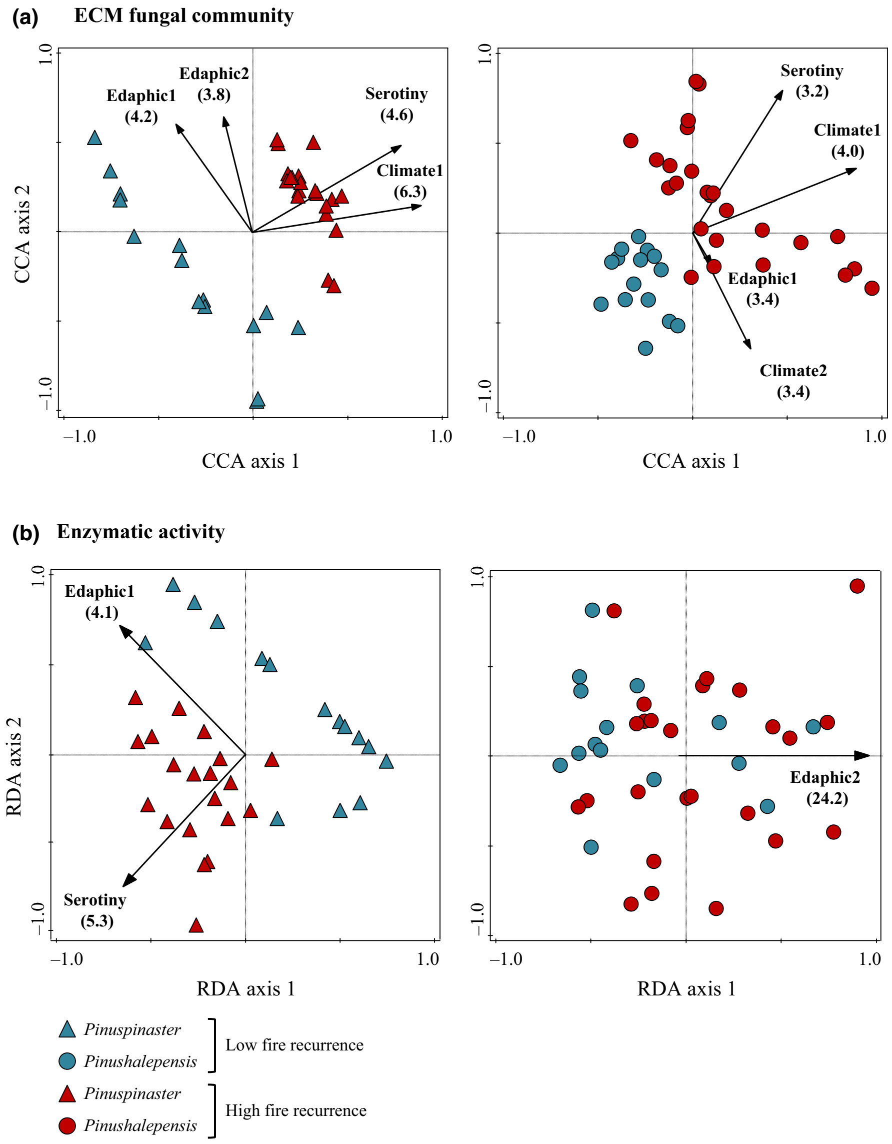

FIGURE 4 (a) Ectomycorrhizal (ECM) fungal community structure and (b) enzymatic activities associated with root tips of *Pinus pinaster* (triangles) and *Pinus halepensis* (circles) forests subjected to low (blue) or high (red) fire recurrence, respectively analysed by canonical correspondence (CCA) and redundancy (RDA) analyses. In all cases, the weight, direction and separate effects of serotiny, climatic and edaphic conditions are represented by vectors (see model details in Table S4). Serotiny degree is the number of closed cones over the total number of cones in the tree. Edaphic and climatic conditions are represented by the first two axes (Edaphic1 and Edaphic2; Climate1 and Climate2) of principal coordinates analyses (PCAs) performed considering the pH, electrical conductivity, and potassium, nitrogen, phosphorus and organic matter content in the first case, and mean temperature, mean precipitation and altitude in the second case (Figure S2). Only significant variables are shown, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ [Colour figure can be viewed at wileyonlinelibrary.com]

Courty et al., 2011, 2016; Schneider et al., 2012). As previously mentioned, laccase activity increased in HiFi populations of both pines. Laccase is an oxidative enzyme related to several processes such as the degradation of recalcitrant C compounds (i.e., lignin), but also to the production of melanin by fungi (Baldrian, 2006; Eisenman et al., 2007). Melanin comprises a group of complex polymers deposited in the cell wall associated with resistance to stresses such as drought (Koide et al., 2014) and moderately resistant to decomposition (Fernandez, Heckman, Kolka, & Kennedy, 2019). It is also possible that in our study sites, changes in traits such as production of melanin by fungi or more litter tannins by trees associated with fire recurrence and climatic stress might have affected OM turnover and feedback cycles, issues that would deserve further analyses. In fact, in a 2-million-year chronosequence, Albornoz et al. (2016) showed strong variation of ECM fungal communities even within the same hosts, attributable not only to short-term fungal edaphic specialization or different inoculum density and composition, but also probably to much longer-term ecosystem-level feedbacks among soil, plants and ECM fungi during pedogenesis.

5 | CONCLUSIONS

Pinus pinaster and *Pinus halepensis* trees growing under a warm and dry Mediterranean climate subjected to historical high fire recurrence hold lower local and regional ECM fungal diversity compared with pines growing in areas where fires are rare. Factors such as fire recurrence, climate and soil properties, in particular pH, explained ECM fungal community composition and their enzymatic functions. Aside from the direct climatic and fire recurrence filtering effect, local adaptation in pine populations to recurrent fires (i.e., increased serotiny) selects for different ECM fungal communities, accompanying different functional responses. A general decline of ECM root-tip enzymatic activity occurred in serotinous *P. pinaster* HiFi populations whereas, for *P. halepensis*, a functional redundancy in LoFi and HiFi areas probably exists. Differences between the ECM fungal communities associated with *P. pinaster* and *P. halepensis* forests was more likely to be related to the bedrock material, overriding the possible effect of the tree species. Although fire is a main factor shaping Mediterranean ecosystems, changes in fire frequency because of predicted warmer and longer dry periods have the potential to affect the plants and their symbionts, altering the structure, functioning and successional dynamics of Mediterranean forest ecosystems. The observed fire recurrence-related structural and functional shifts in ECM fungal communities might have essential implications for the dynamics of Mediterranean pine forests, which should be carefully considered to promote the sustainable management of these vulnerable ecosystems and to maintain their resilience under future climatic scenarios.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

A.R. and M.V. designed the experiment; L.P.I., M.Z.A. and A.R. collected the data; L.P.I. and M.B. performed the bioinformatics analysis, L.P.I. performed the statistical analyses. L.P.I. and A.R. wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

DATA AVAILABILITY STATEMENT

Data have been deposited in the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) as PRJNA324224.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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