



## Influence of stand age and site conditions on ectomycorrhizal fungal dynamics in *Cistus ladanifer*-dominated scrubland ecosystems

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### ABSTRACT

*Cistus ladanifer*-dominated ecosystems are widely distributed in the Western Mediterranean basin and are affected by recurrent fires. Although these scrublands were traditionally considered unproductive, these systems provide significant ecological benefits via mushroom production, which has increased interest in better understanding these ecosystems to restore and promote productivity. We analyzed 48 plots located in Supra- and MesoMediterranean regions in western Spain to assess the soil fungal community and their ecological drivers using ITS2 rDNA Illumina Miseq. The study plots comprised young (early-stage successional stands), middle- (middle-stage successional stands), and late-stage stands. Shannon diversity index values for total fungi were higher under a MesoMediterranean than under a SupraMediterranean climate type, whereas the richness values for ectomycorrhizal (EcM) taxa were higher in late-stage stands than in the younger stands. EcM community composition was influenced by stand age, climatic variables and edaphic parameters. These *C. ladanifer*-dominated ecosystems support diverse fungi, including edible species such as *Boletus*, *Lactarius*, and *Laccaria*, under specific precipitation, temperature, and late-stage stand factor conditions. Although forest management cannot modify temperature and precipitation, management strategies that consider mosaic landscapes to reduce the severity of potential fires and that retain late successional stands could provide suitable habitats for promoting fungal diversity, production, and function in these scrubland systems.

### 1. Introduction

Scrublands dominated by *Cistus ladanifer* L. are distributed throughout the Western Mediterranean basin (Oria De Rueda et al., 2008; Quintela-Sabarís et al., 2011) owing to their historical adaptation to recurrent fires in this region (Quintela-Sabarís et al., 2011). Little attention has been given to the management of *Cistus*-dominated scrublands because they have been deemed unproductive due to their poor soils (Martín-Pinto et al., 2006; Oria De Rueda et al., 2008). However, previous studies have shown that these *Cistus*-dominated scrublands can provide benefits in the form of wild edible mushrooms when properly managed (Hernández-Rodríguez et al., 2015a). The most valuable fungal species associated with this system is *Boletus edulis*, which is a highly prized gastronomic mushroom in many countries (Boa,

2004). Indeed, in 2014, *B. edulis* yields of over 271.10 kg ha<sup>-1</sup> were recorded (Alday et al., 2017). In addition to their economic role, *C. ladanifer*-dominated scrublands play an important ecological role in the recovery of forest stands post-fire by hosting mycorrhizal fungi that are then able to colonize other tree roots as the new stand develops (Hernández-Rodríguez et al., 2015b). The rapid post-fire recovery of *Cistus* plants is favored by the presence of a fungal cohort that can survive the disturbance. Furthermore, symbiotic networks could play a key role in reducing potential soil erosion (Buscardo et al., 2012) in the first rain following a fire in this ecosystem.

As an ecological factor, fire in general affects fungal community composition (Cairney and Bastias, 2007) and decreases sporocarp production (Hernández-Rodríguez et al., 2013) and the abundance of ectomycorrhizal (EcM) fungi (Castaño et al., 2020). In addition to the

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host plant's succession, other factors such as climate and edaphic parameters have been shown to affect fungal communities and their distribution (de Vries et al., 2012; Yergeau et al., 2009). Host plant succession affects the soil fungal community owing to changes in the host's resource requirement and the soil matrix availability (Tian et al., 2017). For example, the growth of the host over time is concurrent with a decrease in soil fertility, as nitrogen and phosphorus become limiting factors (Castaño et al., 2019). This same process occurs during secondary succession, which follows a disturbance event. Severe disturbance leads to distinct effects on fungal communities because it can provide open space for the survival of different fungal species (Chen et al., 2018; Shi et al., 2019) and release nutrients to support the growth of these species (Denslow, 1995). Thus, as we move along the plant succession, fungal species composition changes (Toivanen et al., 2012), with distinct fungi colonizing distinct successional stages of the host plant (Toivanen et al., 2012), such as the EcM fungi (Smith et al., 2002). Thus, a shift from early-stage EcM communities toward late-stage EcM communities would reflect the stand conditions, which can be explained in terms of the fertility of the soil. However, edaphic variables can influence nutrient availability, which is expected to change as the stand develops (Nara et al., 2003). Thus, the survival and growth of the host plant could be supported by the availability of different EcM species in the soil, particularly during the late-stage succession of host plants, such as *C. ladanifer* stands (Hernández-Rodríguez et al., 2013). In this regard, EcM colonizing species can also be differentiated based on their exploration distance at different stages of host development (Geml, 2019).

Stand-replacing wildfires are a major disturbance in Mediterranean ecosystems. Wildfires particularly affect *Cistus*-dominated scrublands (Hernández-Rodríguez et al., 2013; Sanz-Benito et al., 2022) and can alter both the vegetation and soil properties (Grogan et al., 2000), mainly due to intense heating and ash deposition (Peay et al., 2009). Changes in the vegetation and edaphic variables may significantly impact soil fungal communities in these widely distributed ecosystems. Thus, fire can affect the subsequent structure of fungal communities following succession patterns that are mainly driven by the dynamics of post-fire plant communities (Cairney and Bastias, 2007) of *C. ladanifer* (Hernández-Rodríguez et al., 2013). As *Cistus* plants grow after a fire, changes occur in the succession of the associated fungal community (Gassibe et al., 2011). However, the influence of host plant succession and environmental variables on soil fungal community composition in *C. ladanifer*-dominated scrublands have not yet been evaluated. Most of the studies conducted in these ecosystems have been performed at local scales and investigated a limited set of management variables, such as total or partial clearing and prescribed burning (Castaño et al., 2020; Hernández-Rodríguez et al., 2013). However, knowledge of soil fungal communities is important to understand the relationship between sporocarp production levels of valuable species and the amount of mycelium in the soil (Mediavilla et al., 2017). To gain a deeper understanding of the different environmental factors driving fungal communities as well as host plant succession, we should consider a wide range of variables from broad geographic areas. This should enable the development of strategies to manage mosaic landscapes to reduce the severity of potential fires in these widely distributed ecosystems.

In this study, we hypothesized: (1) that total soil fungal diversity would be affected by *C. ladanifer* stand age, resulting in a cumulative increase in richness in late-stage stands. Furthermore, (2) we expected that the EcM fungal community would show changes along *C. ladanifer* stand age gradients that would be related to differences in site conditions, such as climate variables and soil parameters, unlike the saprotrophic taxa. Finally, (3) we also expected that the EcM fungi would be differentiated by their exploration distance, with more abundant extraradical mycelium belonging to the short-distance exploration-type of EcM in mature stands, whereas medium- or short-distance exploration-type EcM would not be affected by *C. ladanifer* stand age. To test these hypotheses, we investigated the effect of *C. ladanifer* stand age, climate type, and edaphic variables on fungal communities present in

the soil. Our specific objectives were, therefore: (i) to understand how the diversity and richness of the total soil fungi and the EcM genera are affected by *C. ladanifer* stand age after fire; (ii) to identify climate and edaphic variables that significantly drive the composition of EcM and other fungal guilds.

## 2. Materials and methods

### 2.1. Study area

The study areas are in the central-west part of Spain: in Luyego in the province of León (LE) and in Mayalde in the province of Zamora (ZA), which have a SupraMediterranean thermotype; and in Calzada de Oropesa in the province of Ávila (AV) and in Aliseda in the province of Cáceres (CC), which have a MesoMediterranean thermotype (Fig. 1). Thermotype classification is mainly based on the winter temperatures in these regions (Rivas-Martínez, 1983). The MesoMediterranean thermotype is defined by winter temperatures ranging between 13 and 17 °C and by frequent frosts in the winter, whereas the summer is hot. Similarly, the SupraMediterranean thermotype is also characterized by temperatures ranging from 8 to 13 °C in winter. Frosts and snow are common during the winter. According to the thermotype classification of Rivas-Martínez et al. (2011), both the Meso- and SupraMediterranean study areas are characterized by a Mediterranean climate (i.e., a dry season of at least two months in the summer, with most of the precipitation falling between October and February at all sampling sites. The mean annual precipitation at the sites located in the SupraMediterranean provinces is 707 mm at Luyego (LE) and 464 mm at Mayalde (ZA), while the mean annual temperatures are 9.5 °C and 11.3 °C, respectively. The mean annual precipitation at the sites located in the MesoMediterranean provinces is 677 mm at Calzada de Oropesa (AV) and 518 mm at Aliseda (CC), while the mean annual temperatures are 14.9 °C and 16.1 °C, respectively. Climatic variables for all study sites were obtained from nearby meteorological stations.

The selected study areas are dominated by *Cistus ladanifer* vegetation and are characterized by a wide range of elevations ranging from 300 m above sea level at the sampling sites in the south to 1080 m above sea level in the north. Paleozoic metamorphic (slate and quartzite) and plutonic (granite) rocks dominate the landscape, except at the Mayalde (ZA) site, where Tertiary sands are predominant.

### 2.2. Experimental design, soil sampling, and molecular work

In May 2019, we established plots at the four study sites to evaluate the influence of edaphic variables and stand age conditions on fungal communities. The selected study sites comprised three different stand ages. Information regarding the age of the stands was obtained from the Regional Forest Management Services. The age classes were early successional stage stands (4–6 years), middle-stage stands (10–12 years), and late-stage successional stands (20–22 years). Thus, our experimental design was composed of four sites (AV, LE, CA, and CC), with four blocks per site. The blocks were located 2 to 3 km apart and the area of each block ranged from 1.5 to 2 ha. Each block comprised a plot of each of the three stand age groups, with each plot measuring 50 m × 50 m (i.e., 4 sites × 4 blocks per site × 3 sampling plots per block, i.e., 48 sampling plots in total). Soil samples were collected in June 2019. In total, 15 soil samples were collected from each plot using an auger to a depth of 20 cm, including the organic and mineral layers. Soil samples were pooled per plot to create one composite soil sample and to preserve the heterogeneity of the terrain. After collection, samples were dried at room temperature until a constant weight was obtained and then sieved (1 mm “mesh”). A subsample of each plot was stored at –20 °C for molecular analysis. Chemical analyses were performed according to Sparks et al. (1996) to determine the soil pH (water-based), dry matter (%), total phosphorus (P), total carbon (C), and total nitrogen (N) contents of the soil samples (Table 1).

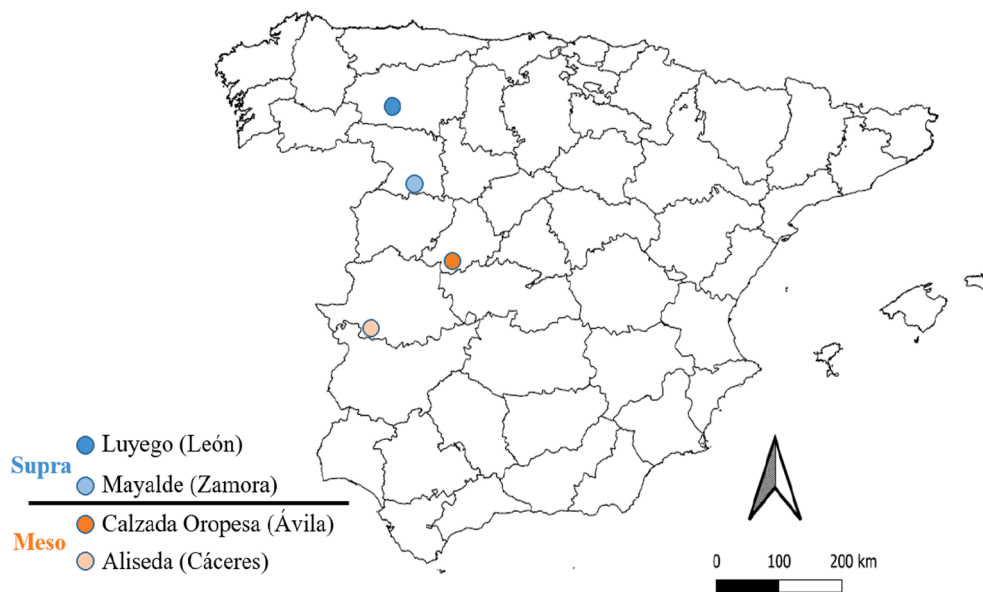


Fig. 1. A map of the four sampling localities (indicated by colored dots) in the central-west part of Spain.

Table 1

Mean chemical properties of soils. Different lowercase letters indicate significant differences among sampling sites, bioclimate types, or stand age based on LME and Tukey's HSD test. The numbers in parentheses are the standard error.

Soil parameters	Sites				Bioclimate		Stand age		
	León	Zamora	Ávila	Cáceres	Supra	Meso	Early	Middle	Late
pH	4.61(5.49)b	4.96(5.72)a	5.11(6.24)a	4.95(5.65)a	4.74(5.63)a	5.02(5.23)b	4.92(5.61)a	4.76(5.49)a	4.76(5.51)a
P Olsen (mg/kg)	16.02(0.08)ab	5.28(0.20)b	23.95(3.29)a	5.23(0.08)b	11.12(0.95)a	15.42(2.69)a	9.46(0.84)a	16.61(2.86)a	12.25(1.79)a
% N	0.16(0.01)a	0.07(0.01)b	0.15(0.01)a	0.13(0.01)a	0.11(0.01)a	0.14(0.01)a	0.13(0.01)a	0.14(0.01)a	0.13(0.01)a
% C	2.48(0.14)a	0.95(0.05)b	2.64(0.14)a	2.05(0.10)a	1.71(0.15)a	2.35(0.13)b	1.90(0.12)a	2.12(0.15)a	2.23(0.16)a
% Dry matter	98.88(0.05)b	99.56(0.02)a	98.54(0.04)c	99.07(0.03)b	99.22(0.06)a	98.81(0.06)b	99.03(0.06)a	99.01(0.06)a	99.00(0.07)a

Note: the León and Zamora sites have a SupraMediterranean bioclimate (Supra) while Ávila and Cáceres have a MesoMediterranean bioclimate (Meso). The pH values are provided by the 95% Confidence intervals calculated from  $[H^+]$  and then back-transformed to pH given that  $pH = -\log_{10}[H^+]$ .

### 2.3. Molecular analysis

We used the Qiagen PowerLyzer® PowerSoil™ DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) to extract DNA from 0.25 g of soil per sample according to the manufacturer's protocol. PCR amplifications employed primer sets that targeted ITS4 and the fITS7. PCR reactions of each sample were carried out in triplicate to minimize PCR biases. PCR reactions were performed in 20  $\mu$ L reaction volumes containing 11.22  $\mu$ L of Modified Quantization (MQ) water, 1.60  $\mu$ L of DNA template, 2.00  $\mu$ L of 10  $\times$  buffer, 1.40  $\mu$ L of  $MgCl_2$  (50 mM), 1.60  $\mu$ L of dNTPs (10 mM), 0.50  $\mu$ L of Bovine Serum Albumin (2%), 0.80  $\mu$ L of reverse and forward primers (10  $\mu$ M), and 0.08  $\mu$ L of Platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA). The following PCR conditions were used: an initial denaturation step at 95  $^{\circ}$ C for 5 min; then 37 cycles of 95  $^{\circ}$ C for 20 s, 56  $^{\circ}$ C for 30 s, and 72  $^{\circ}$ C for 1.5 min; ending with one cycle of 72  $^{\circ}$ C for 7 min. To amplify the ITS2 rDNA region (ca. 250 bp), we used the forward primer fITS7 (Ihrmark et al., 2012) and the barcoded reverse primer ITS4 (White et al., 1990). Sample-specific Multiplex Identification DNA tags were used to label the ITS4 primer. Each set of PCR replicates also included a negative control comprising MQ water instead of DNA that underwent PCR under the same experimental conditions and was shown to be amplicon-free on a gel. Sequencing was performed using an Illumina MiSeq platform (BaseClear BV).

### 2.4. Bioinformatic analysis

We used cutadapt (Martin, 2011) to trim low-quality ends and

merged the paired reads using USEARCH v.10.0.240 (Edgar, 2010). Cutadapt was set with a quality score of 5 and a minimum sequence length of 200 bp. Primer pairs (ITS4 and fITS7) were trimmed and sequences with an expected error of  $>1$  were removed. The remaining sequences were merged into unique sequence types on a per-sample basis using USEARCH v.8.0 (Edgar, 2010) while preserving read counts. High-quality sequences were grouped with USEARCH at 97% sequence similarity to generate operational taxonomic units (OTUs) while simultaneously excluding sequences representing OTUs with  $<70\%$  similarity or  $<200$  bp pairwise alignment length to a fungal sequence. The sequences were assigned to taxonomic groups based on pairwise similarity searches against the curated UNITE + INSD fungal ITS sequence database (version v.8.0), which contains identified and unidentified sequences assigned to species hypothesis groups defined based on dynamic sequence similarity thresholds (Köljalg et al., 2013). Functional groups were assigned to each OTU using Fungal Traits (Pölme et al., 2020). Further classification of EcM fungi as either short-distance exploration, long-distance exploration, or mixed-distance exploration types was performed following the criteria proposed by Geml (2019) and data published by Agerer (2006), Tedersoo and Smith (2013), and the DEEMY database (<https://deemy.de>). Finally, edible fungi were identified using specialist books and fungal guides, such as Gerhardt et al. (2000) and Moreno and Manjón (2010), to assess the commercial importance of the fungal communities in the study areas.

### 2.5. Statistical analysis

Krona charts were used to visualize the taxonomic distribution of all

fungi and guilds based on OTU richness following Tedersoo et al. (2020). To normalize the OTU table for subsequent statistical analysis, we rarefied the number of high-quality fungal sequences (35,404 reads). After standardizing the environmental variables and transforming the OTU rarefied matrix using the Hellinger transformation method, canonical correspondence analysis (CCA) was performed to analyze the fungal communities associated with the different stand types based on their successional stage. For this purpose, when the length of the extracted gradient was <3 SD units, we used redundancy analysis (RDA) (Ter Braak, 1986) to assess the correlation between environmental variables and the fungal composition. The ordination was based on the abundance of each fungal group in each sample using CANOCO version 5.0 (Smilauer and Lepš, 2014). The forward selection was used to select significant explanatory variables and only those significant at the  $p < 0.05$  level were included in the models. Bonferroni correction was also used after including each environmental variable to be more restrictive in the selection of variables. The statistical significance of environmental variables was calculated using the Monte Carlo permutation test (499 permutations). The effect of stand age on phyla and EcM species within communities, including the effect of the blocks within each site, were analyzed using a PerMANOVA based on 999 permutations using the *adonis* function in the *vegan* package. A similarity percentages (SIMPER) routine was run to identify the fungal species responsible for the dissimilarity in the community structures (Parravicini et al., 2010). The analysis was performed using PAST software (Hammer et al., 2001). In addition, we used Mantel tests based on Pearson's correlation to determine the influence of climate and soil variables on the soil fungal community using Bray–Curtis distance for the rarefied OTU total matrix and Euclidean distance for the scaled environmental parameters. Data were scaled using R (R Core Team, 2019) when needed to normalize data for analysis. Significant effects of thermotypes and stand age were specifically tested by Linear Mixed Effects (LME) models (Pinheiro et al., 2016) with a nested design, where blocks were nested in site and included as a random factor. Stand age and thermotype were defined as fixed factors. LME models were used to prevent false-positive associations due to the relatedness structure in the sampling. When significant effects were found, we performed Tukey post hoc specific contrasts for stand ages as just two thermotypes were studied. We determined any preferences of individual EcM fungal OTUs for a specific stand type using indicator species analyses (Dufren, 1997). This analysis was performed using the *interspecies* package (Cáceres and Legendre, 2009).

### 3. Results

#### 3.1. Sequencing output and fungal community composition

A total of 2,258,090 sequencing reads, with a minimum of 35,404 reads per sample, passed quality filtering, representing 2057 fungal OTUs and 12 fungal phyla (Fig. 2). Ascomycota and Basidiomycota were the dominant phyla in all sample plots, accounting for 90% of sequences. Among the guilds, EcM fungi (44.1%) and saprotrophs (10%) were the most dominant. Other groups, including arbuscular mycorrhizal fungi, parasitic fungi, and animal pathogens, represented <1% of sequences. However, we were unable to assign a guild to 37.14% ( $N = 765$ ) of OTUs. The taxonomic distribution of all fungi recognized at the phylum level and of guild groups is shown in Fig. 2.

We found significant differences in the distribution of Basidiomycota phyla groups between the two climate types ( $p < 0.05$ ; Table 2). For Basidiomycota significantly higher abundance values were obtained in plots with a SupraMediterranean climate type. However, only the abundance of the Mucoromycota phyla was affected by the stand age in this study (Table 2).

#### 3.2. Fungal richness and diversity according to stand age and climate

The total fungi Shannon diversity index (H) was significantly

affected by climate ( $F = 4.06, p = 0.049$ ) and by stand age ( $F = 3.05, p = 0.050$ ). Higher H values were obtained for fungal communities in plots with a MesoMediterranean climate type and in plots with mature stands (Fig. 3). However, no differences were found when comparing the total fungal richness values of plots with different climate types or stand ages (Fig. 3).

Analysis of specific guilds revealed that none of the guilds was significantly affected by climate type ( $p > 0.05$ ; Table 3). However, stand age had a significant effect on EcM fungi ( $p < 0.0001$ ; Table 3), with higher richness values obtained for the late-stage successional stands ( $p < 0.05$ ; Fig. 4). The richness of EcM short- and long-exploration types increased with stand age ( $p < 0.05$ ). There was no change in EcM exploration types or of saprotroph richness under different climate types ( $p > 0.05$ ; Fig. 4).

#### 3.3. Environmental factors affecting fungal composition

The CCA of phylum-level taxa followed by perMANOVA analyses confirmed that soil fungal communities at the four studied sites differed ( $F = 6.06, R^2 = 0.29, p = 0.001$ , Fig. 5). The SIMPER analysis also identified fungal OTUs at the phylum level that were responsible for the differences between the sites. Mucoromycota and Ascomycota were the dominant phyla and were generally influential across the pairwise comparisons (Table S1). The cumulative contribution of the most influential phyla for the dissimilarity between the studied sites is provided (Table S1).

The climatic and edaphic parameters were also correlated with soil fungal community composition ( $p < 0.05$ ; Fig. 5). A Mantel test confirmed that climatic variables aggregately had a stronger effect on fungal community structure ( $r = 0.3076, p = 0.001$ ) than the grouped edaphic variables ( $r = 0.07184, p = 0.012$ ). Specifically, the mean annual temperature (Temp), mean annual precipitation (Prec), pH, and phosphorus (P) showed a highly significant influence on the composition of the fungal community at the phylum level (Fig. 5; Table 4). Only climatic parameters (i.e., temperature and precipitation) influenced total fungal guild composition (Table 4).

RDA of the total EcM OTUs followed by perMANOVA analyses confirmed that EcM communities differed among the stands ( $F = 3.39, R^2 = 0.13, p = 0.001$ , Fig. 6A). The SIMPER analysis also identified EcM genera responsible for the difference between the stands. Among the different genera, *Cortinarius*, *Russula*, *Hebeloma*, and *Amanita* were identified as the dominant genera that were generally influential across the pairwise comparisons. The cumulative contribution of the most influential EcM genera for the dissimilarity between stands is indicated in Table S2. Explanatory variables such as stand age, climate variables, and edaphic parameters had a significant influence on EcM fungal community composition ( $p < 0.05$ ; Fig. 6A). The Mantel test confirmed that climatic variables (precipitation and temperature) aggregately had a higher effect on EcM fungal community structure ( $r = 0.4817, p = 0.001$ ) than the grouped edaphic variables (pH, N, C, and P) ( $r = 0.3786, p = 0.001$ ).

Similarly, RDA followed by perMANOVA of EcM OTUs according to mycelial exploration type also showed that EcM exploration types differed among the studied stands ( $F = 6.52, R^2 = 0.23, p = 0.001$ , Fig. 6B). The SIMPER analysis also identified the EcM exploration types responsible for the difference between the stands: the short-distance exploration type made the greatest contribution to dissimilarity across all stands (Table S3). Stand age ( $r = 0.27, p = 0.004$ ), N ( $r = 0.096, p = 0.038$ ), and C ( $r = 0.4, p = 0.002$ ) contributed significantly to the variation in EcM exploration types (Fig. 6B) among stands. Moreover, valuable edible fungal species were distributed differently among the host stand ages. The genera *Boletus*, *Tricholoma*, and *Lactarius* were associated with the late or middle successional stages, whereas *Amanita* was associated with the early successional stage (Fig. 6A).

A total of 49 EcM genera were associated with *C. ladanifer* stands. However, we found indicator EcM genera only in the mature and young



**Table 2**  
Influence of climate or stand age on the abundance of specific fungal phyla. Values in bold indicate significant effects ( $p < 0.05$ ).

Phylum	Climate		Stand age	
	F	p-value	F	p-value
Ascomycota	1.01	0.42	1.79	0.18
Basidiobolomycota	0.96	0.33	1.89	0.16
Basidiomycota	<b>4.87</b>	<b>0.03</b>	0.04	0.96
Calcarisporiellomycota	1.51	0.34	0.26	0.76
Chytridiomycota	0.000	0.97	1.09	0.34
Entorrhizomycota	0.96	0.43	1.29	0.28
Kickxellomycota	0.16	0.72	2.29	0.11
Mortierellomycota	0.13	0.75	2.59	0.08
Mucoromycota	0.66	0.50	<b>4.13</b>	<b>0.02</b>
Olpidiomycota	2.03	0.17	0.72	0.49
Rozellomycota	2.54	0.24	1.50	0.23
Zoopagomycota	0.19	0.65	1.29	0.28

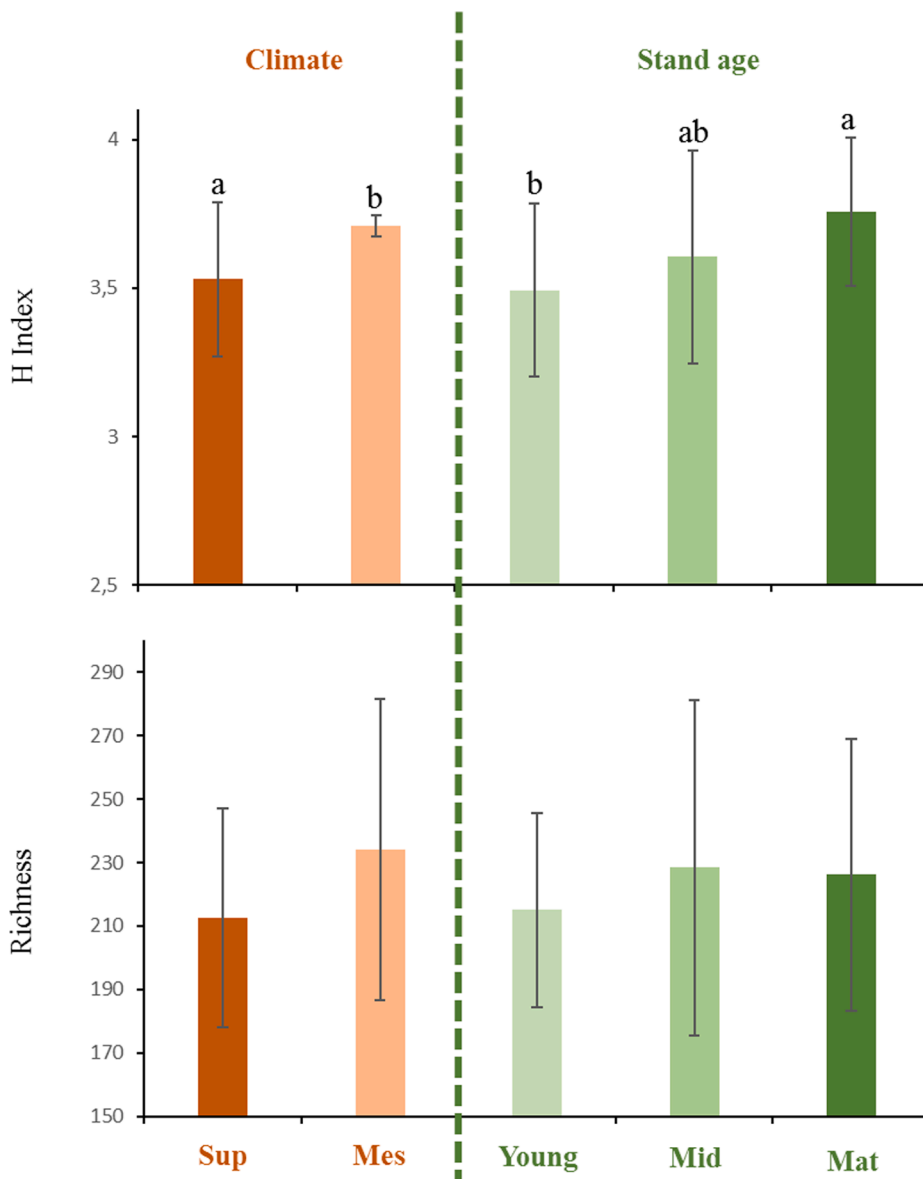
stands in our study area (Table S4). Among the total EcM genera, only three (*Inocybe*, *Tricholoma*, and *Thelephora*) were significant indicator species ( $p < 0.05$ ) of mature *C. ladanifer* stands, while four EcM genera (*Amanita*, *Hebeloma*, *Scleroderma*, and *Pisolithus*) were indicator species

associated with early successional stands. When considering the climate region, the genera *Pisolithus*, *Terfezia*, and *Hysterangium* were significantly ( $p < 0.05$ ) associated with the MesoMediterranean environment type, whereas *Boletus*, *Lactarius*, and *Laccaria* were associated with the SupraMediterranean environment (Table S4).

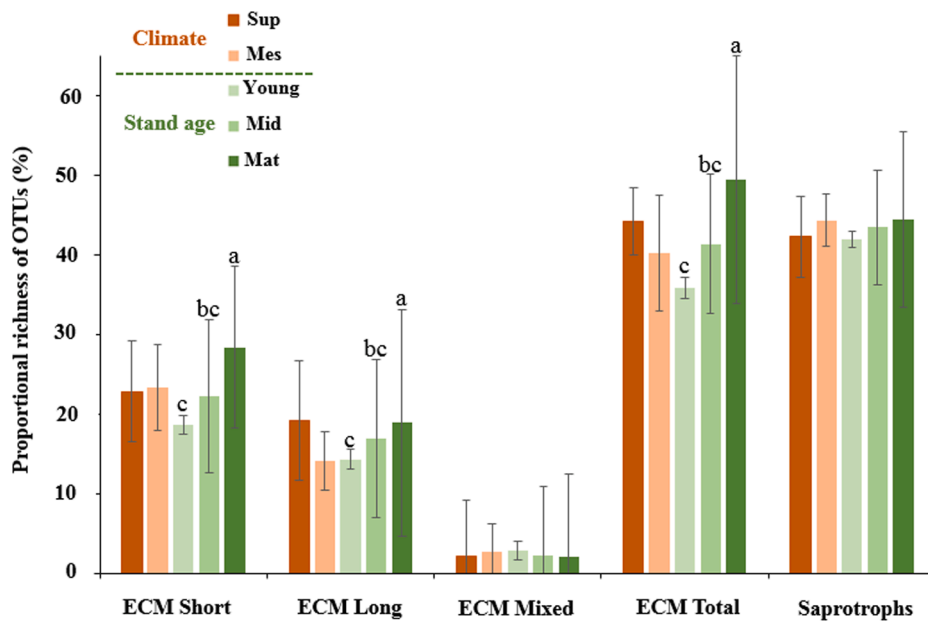
Finally, indicator species analysis of edible EcM at the species level

**Table 3**  
Influence of climate or stand age on the richness of specific fungal guilds. Values in bold indicate significant effects ( $p < 0.05$ ).

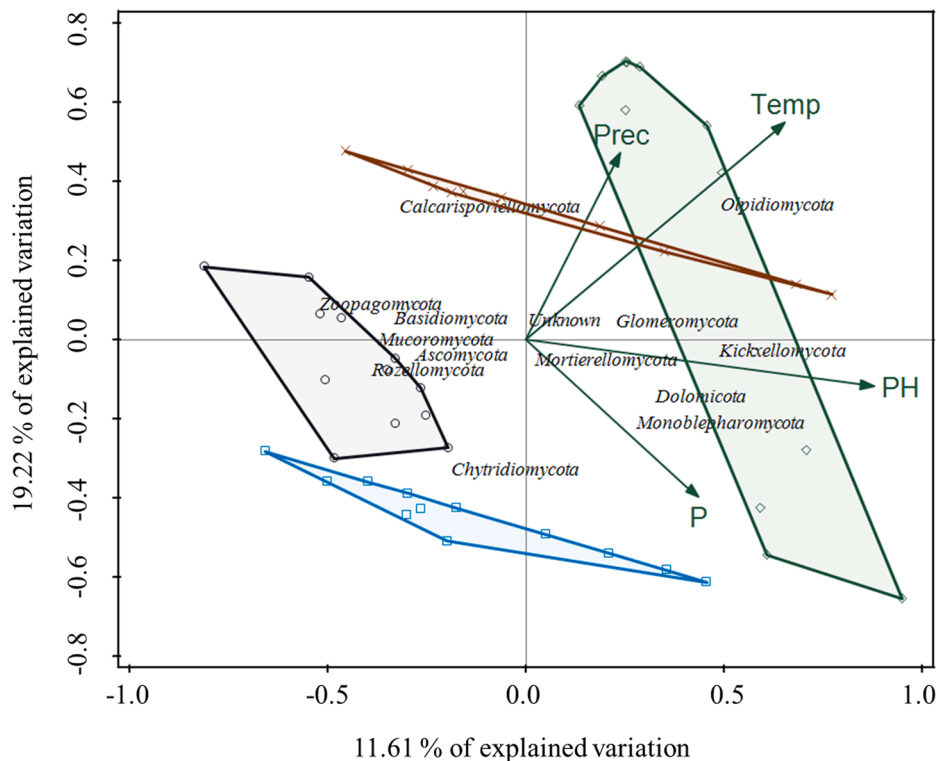
Guilds	Climate		Stand age	
	F	p-value	F	p-value
Animal pathogens	6.79	0.12	0.17	0.84
Arbuscular mycorrhizae	6.13	0.13	1.59	0.22
Biotroph generalists	0.46	0.56	0.19	0.82
Ectomycorrhizal fungi	0.87	0.44	<b>11.88</b>	<b>0.00</b>
Fungal parasites	0.43	0.57	1.30	0.28
Lichenized fungi	0.99	0.33	1.72	0.19
Litter decomposers	0.99	0.42	0.95	0.39
Plant pathogens	2.18	0.14	0.52	0.59
Saprotroph generalists	0.53	0.59	0.03	0.86



**Fig. 3.** Shannon H index and richness of the total soil fungal community detected in plots with a SupraMediterranean (Sup) climate type or a MesoMediterranean (Mes) climate type (A) or in young, middle- (Mid) or late-stage (Mat) stands (B). Means were compared using Tukey's HSD tests, with different lowercase letters denoting significant differences between means ( $p \leq 0.05$ ). The error bar shown for each mean value is the standard deviation of the mean. The absence of letters above the richness mean values indicates that there is no significant difference between means.



**Fig. 4.** Richness values of EcM mycelial exploration types and saprotrophic fungi detected in plots with a SupraMediterranean (Sup) climate type or a Meso-Mediterranean (Mes) climate type and in early- (Young), middle- (Mid), or late-stage (Mat) stands. EcM mycelial exploration types: EcM short, short-distance exploration type; EcM Long, long-distance exploration type; and EcM Mixed, mixed-distance exploration type. Means were compared using Tukey's HSD tests, with different lowercase letters denoting significant differences between means ( $p \leq 0.05$ ) for each grouped set of data according to guild type and climate/stand age. The error bar shown for each mean value is the standard deviation of the mean. The absence of letters above mean values indicates that there is no significant difference between means.



**Fig. 5.** Canonical correspondence analysis of phyla detected in *Cistus ladanifer* stands at four sites with either a SupraMediterranean (SUP) climate type or a MesoMediterranean (ME) climate type. Precipitation (Prec), temperature (Temp), pH, and phosphorus (P) are constrained significant parameters.

revealed that none of the indicator species were significantly associated with stand age. However, we found that *Terfezia pseudoleptoderma* was significantly associated with the MesoMediterranean thermotype ( $p = 0.006$ ), while *Cortinarius purpurascens* ( $p = 0.001$ ) and *Boletus edulis* ( $p = 0.005$ ) were indicator species for the SupraMediterranean thermotype. These species showed at least 98% similarity to the reference sequences (Table S5).

#### 4. Discussion

In this study, our initial hypothesis that the total soil fungal diversity and richness would be affected by the successional stage of the *Cistus ladanifer* stands after the fire, was supported by our findings, assuming that the fungal species keep colonizing the habitat, resulting in a cumulative increase in richness in stands at late-stage succession (Castaño et al., 2019). Our findings differed from those reported for a recent chronosequence study in a European temperate forest that covered 137 years, which found no relationship between total fungal diversity,

**Table 4**

Significance of explanatory variables from constrained multivariate analysis, CCA for phyla, and all categorized guilds. The Hellinger-transformed fungal community was considered. Values in bold indicate significant effects ( $p < 0.05$ ).

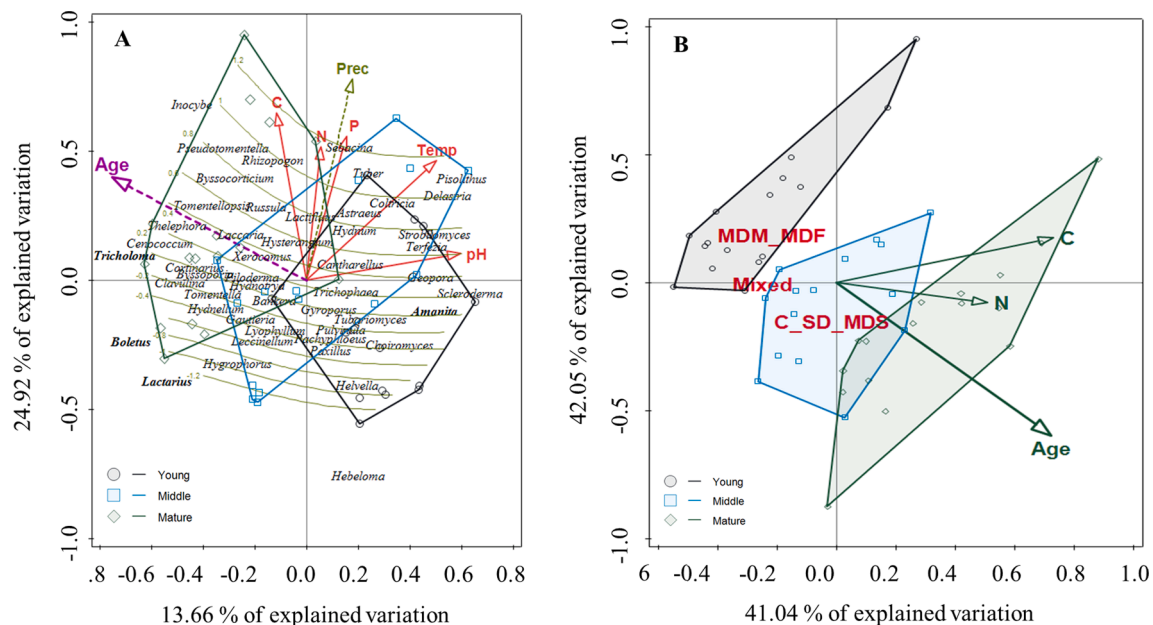
	Variable	Explains %	Contribution %	pseudo-F	p-value
<b>Phyla</b>	pH	9.3	32.0	4.6	<b>0.014</b>
	Temperature	6.1	20.8	3.1	<b>0.014</b>
	Phosphorus	3.9	13.5	2.1	<b>0.002</b>
	Precipitation	4.7	16.1	2.6	<b>0.042</b>
<b>Guilds</b>	Temperature	11.6	35.8	5.9	<b>0.002</b>
	Precipitation	11.1	28.7	5.6	<b>0.010</b>

richness, and stand age (Odrizola et al., 2020). In our study, the effect of stand age on the fungal community could be due to the strong dominance of EcM fungi. As expected, we found a greater presence of mycorrhizal fungi in late-stage stands than in the younger stands: 49.38% of the total OTUs found in late-stage stands were EcM species. This also supports our hypothesis that the effect of stand age on fungal communities would be more pronounced among mycorrhizal fungi, which may be related to changes in soil fertility and increasing tree root cover as stands age (Castaño et al., 2019). Thus, the greater dependency of trees in late-stage stands on their fungal symbionts could compensate for the decrease in soil fertility and increase in plant nutrient stress over time (Castaño et al., 2019; Read and Perez-Moreno, 2003). Under such conditions, EcM fungi could also play a role in protecting their hosts from biotic and abiotic stresses (Agerer, 2001; Defrenne et al., 2019). In addition, the effect of stand age was observed for both short-distance and long-distance exploration-types of EcM species. Perhaps in *Cistus*-dominated scrubland systems, where root systems are very dense, this ecological concept may be less relevant than in woodland systems, where the low root density of mature stands leads to the greater dominance of the long-distance exploration-type of EcM fungi (Geml, 2019), which therefore provide a greater benefit to the ecosystem than the short-distance exploration type.

Our soil analyses also showed that the richness of saprotrophic fungi did not change with stand age. Communities of saprotrophic fungi are known to be less influenced by stand age than by substrate type, which is primarily influenced by the plant community (Gebauer and Taylor,

1999) and, therefore, could be influenced by abiotic factors (Zakaria and Boddy, 2002). Consequently, saprophytic species tend to be more stable over successional stages and are more influenced by temperature or soil humidity (Sysouphanthong et al., 2010). In these *Cistus*-dominated scrubland ecosystems, saprophytic fungi found suitable niches throughout the successional cycle. In the early stages of succession, the absence of vegetation affects the abundance of plant-dependent mycorrhizal fungi. During this time, saprophytic fungi take advantage of the lack of competition to spread rapidly (Hernández-Rodríguez et al., 2013). In the Mediterranean ecosystem, organic matter rapidly accumulates on the soil surface and, due to the lack of overlap between peak humidity and temperature, mineralization is extremely slow (García and Hernández, 1996; Rodeghiero et al., 2011). In addition, the high plant density of *Cistus*-dominated ecosystems protects the soil from direct sunlight, which generates adequate humidity for the development of these saprophytic fungi throughout the entire cycle of these very particular systems (Mediavilla et al., 2021).

Our findings are in agreement with those reported by previous studies of various biomes that highlighted the role of climatic and edaphic variables in total fungal community composition (Canini et al., 2020; Tedersoo et al., 2014; Tedersoo et al., 2021; Větrovský et al., 2020). Although in this study we found climate types affected the Basidiomycota richness, the general phyla composition were moderately associated climate variables such as temperature and rainfall, as reported by Tedersoo et al. (2014) and Newsham et al. (2016). This is because higher air temperature and soil moisture levels enhance fungal activities in the soil, enabling them to switch from survival to growth strategies (Newsham et al., 2016). Furthermore, edaphic variables, such as pH and phosphorus, also affect fungal communities, as reported by Lauber et al. (2008) and Nilsson et al. (2007). In this study, we also observed a relationship between pH and EcM fungi rather than other guilds such as decomposers or pathogenic fungi. This finding is in agreement with previous studies that indicated a direct relationship between pH and EcM fungi (Canini et al., 2019) and a negative relationship with other functional groups such as saprotrophs, plant and animal pathogens, and mycoparasitic fungi (Canini et al., 2019). This is because EcM species can play an important role in plant growth in acidic soils, such as *Cistus*-dominated scrublands, where the availability of essential nutrients is low (Mengel and Kirkby, 2001). Furthermore,



**Fig. 6.** Redundancy analysis of EcM genera (A) and according to EcM exploration type (B) related to explanatory climatic and edaphic variables grouped by stand type. Isolines represent precipitation using a generalized additive model-fitting method ( $F = 27.0, p < 0.000$ ). EcM exploration type: C\_SD\_MDS, contact/short-distance/medium-distance smooth with hydrophilic hyphae; MDM\_MDF, medium-distance mat/medium-distance fringe with hydrophobic hyphae.



phosphorus uptake from the soil is facilitated indirectly via mycorrhizal symbiosis, where the host plant obtains phosphorus primarily from the fungal partner, while the fungus benefits from plant-derived reduced carbon (Johri et al., 2015). Similarly, our finding that temperature and precipitation explain some of the variations in EcM assemblages was similar to the findings reported by Pena et al. (2017). However, the aggregate effect of climate on the structure of the EcM fungal community in this study could also be related to site effects, such as the poor soil conditions and recurrent fires, that assisted the co-evolution of the host species (*C. ladanifer*) and fungal symbionts (Defrenne et al., 2019) in the study area.

The indicator species analysis revealed that EcM fungal genera have a preference for a particular stand successional stage, suggesting that stand succession can influence the structure of the EcM fungal community in a *Cistus*-dominated ecosystem. For example, species belonging to the genera *Amanita*, *Hebeloma*, *Scleroderma*, and *Pisolithus* were associated with early successional stands. Although these genera are characteristic of late-stage species in *Pinus* stands (Chu-Chou and Grace, 1982; Visser, 1995), when these species are associated with *Cistus* spp., they behave as early-stage mycorrhizal species. This supports findings reported by Hernández-Rodríguez et al. (2013) who found sporocarps of these species when *C. ladanifer* was at the early stages of development. Similarly, we found that *Inocybe*, *Tricholoma*, and *Thelephora* were associated with late-stage stands. However, the presence of most of these species has been reported at both the early and late stages of *C. ladanifer* development (Hernández-Rodríguez et al., 2013), indicating that these genera are also multi-stage species that might be able to fruit under different ecological conditions and that they have wide substrate requirements. Furthermore, the network of mycorrhizal mycelium could also play a role in reducing erosion that could potentially occur when the first rain falls after a fire. Thus, the conservation and rehabilitation value of *Cistus* scrubland, which is rich in a range of valuable species, along with the development of the stands, is considerable due to the short life cycle (18–20 years) of *C. ladanifer*. However, when stands of this species wither and die, they create a very high risk of forest fires (Oria De Rueda et al., 2008). Thus, the management of *Cistus* scrublands should involve measures to prevent fuel continuity in the system. In this sense, mosaic stand structure management, including young, intermediate, and mature *C. ladanifer* stands simultaneously, may help to conserve fungal richness and diversity and facilitate the production of some highly prized and sort after edible species, while preventing the occurrence of wildfires in these areas by reducing fuel loads.

In the absence of fire, *Cistus*-dominated scrublands will be progressively occupied by other species such as *Quercus ilex* L. or *Quercus pyrenaica* Willd. (Hernández-Rodríguez et al., 2013). Because of this, consideration of fire-prevention treatments is also vital as *Cistus*-dominated ecosystems can act as a bridge, providing mycorrhizal inoculum that can colonize tree roots as a new stand develops or infecting new roots in younger stands during the recovery of forest stands after a fire, thereby, enabling the system to produce greater quantities of mycorrhizal edible sporocarps. In addition, the dispersion and sharing of mycorrhizal species from nearby host forests, such as *Quercus* stands, with *Cistus*-dominated stands, or vice versa, should be considered in the management strategy of a *Cistus*-dominated system as the presence of such forests, which act as alternative hosts or reservoir species, enables a wide diversity of fungal species to disperse to or to persist in this ecosystem (Tomao et al., 2017). Therefore, after a fire, managers of these ecosystems can choose to keep scrublands, to maintain the fungal richness and diversity associated with it, or to use *C. ladanifer* as a tool to provide fungal propagules for the establishment of a new forest stand in the area (Hernández-Rodríguez et al., 2015b).

## 5. Conclusions

We observed a strong influence of stand age on the general diversity indices and on the richness of the studied fungal community, including

the EcM fungal community. The fungal community composition was primarily driven by climatic and edaphic factors, whereas stand age only had a weak influence on the fungal community. Analysis at the phylum level revealed that edaphic variables such as phosphorus and pH significantly influenced the composition of the fungal community. Ordination analysis of EcM genera showed that stand age and climate type had a significant driving effect on this community. Analysis of the EcM exploration type also revealed an effect of stand age, with a greater richness of short-distance exploration-type taxa in the oldest stands. Finally, some genera, such as *Inocybe*, *Tricholoma*, and *Thelephora*, were significantly associated with mature stands, whereas *Amanita*, *Hebeloma*, *Scleroderma*, and *Pisolithus* were associated with early successional stands. Furthermore, the genera *Pisolithus*, *Terfezia*, and *Hysterangium* were significantly associated with the MesoMediterranean environment type whereas *Boletus*, *Lactarius*, and *Laccaria* were associated with the SupraMediterranean environment. Remarkably, *C. ladanifer*-dominated scrublands support diverse fungi, including highly regarded edible species, such as *Boletus* and *Lactarius*, under specific climate, soil, and stand factor conditions. These findings suggest that *C. ladanifer* scrublands may play an important role in the recovery of forest stands after a fire by providing mycorrhizal inoculum that can colonize tree roots as new stands develop. Although management cannot modify climate parameters such as precipitation and temperature, management strategies that consider mosaic landscapes to reduce the severity of potential fires in these widely distributed scrubland ecosystems could provide suitable habitats for promoting fungal diversity, production, and function. Furthermore, the retention of late-successional stands as part of a mosaic landscape management approach would have important implications for scrubland floor microhabitats, such as soil fertility improvement, which are important for macrofungal occurrence and production in *Cistus*-dominated scrublands. Our findings could serve to guide future studies in many other countries with similar contexts to optimize *Cistus*-dominated ecosystems that are affected by recurrent fires.

## CRedit authorship contribution statement

**Pablo Martín-Pinto:** Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing. **Juan Andrés Oria-de-Rueda:** Supervision, Methodology. **Tatek Dejene:** Investigation, Methodology, Writing – review & editing. **Olaya Mediavilla:** Investigation, Methodology. **María Hernández-Rodríguez:** Investigation, Methodology. **José A. Reque:** Supervision. **Ignacio Sanz-Benito:** Writing – review & editing. **María Santos:** Investigation, Methodology. **József Geml:** Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foreco.2022.120340>.

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