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1 **Soil microclimate changes affect soil fungal communities in a**  
2 **Mediterranean pine forest**

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36

## 37 **Summary**

- 38 • Soil microclimate is a potentially important regulator of the composition of plant-  
39 associated fungal communities in climates with significant drought periods. Here,  
40 we investigated spatio-temporal dynamics of soil fungal communities in a  
41 Mediterranean *Pinus pinaster* forest in relation to soil moisture and temperature.
- 42 • Fungal communities in 336 soil samples collected monthly during a year from 28  
43 long-term experimental plots were assessed by PacBio sequencing of ITS2  
44 amplicons. Total fungal biomass was estimated by analysing ergosterol.  
45 Community changes were analysed in the context of functional traits.
- 46 • Soil fungal biomass was lowest during summer and late winter and highest  
47 during autumn, concurrent with a greater relative abundance of mycorrhizal  
48 species. Intra-annual spatio-temporal changes in community composition  
49 correlated significantly with soil moisture and temperature. Mycorrhizal fungi  
50 were less affected by summer drought than free-living fungi. In particular,  
51 mycorrhizal species of the short-distance exploration type increased in relative  
52 abundance under dry conditions, whereas species of the long-distance exploration  
53 type were more abundant under wetter conditions.
- 54 • Our observations demonstrate a potential for compositional and functional shifts  
55 in fungal communities in response to changing climatic conditions. Free-living  
56 fungi and mycorrhizal species with extensive mycelia may be negatively affected  
57 by increasing drought periods in Mediterranean forest ecosystems.

58 *Keywords: climate, drought, ergosterol, fungal biomass, fungal community,*  
59 *mycorrhizal.*

## 60 **Introduction**

61 Soil fungi are essential drivers of organic matter dynamics and nutrient release and  
62 uptake in coniferous forest ecosystems. However, climate changes, such as warming or  
63 increased drought, may alter the composition of soil fungal communities (Fernandez *et*  
64 *al.*, 2016; Solly *et al.*, 2017; Hartmann *et al.*, 2017). In Mediterranean forest  
65 ecosystems, drought stress and low nutrient availability are important determinants of  
66 functional and structural traits of plants (e.g. sclerophylly and low growth rate; Sardans  
67 & Peñuelas, 2013). However, expected increases in temperature and the unprecedented  
68 duration and intensity of drought events could exceed the tolerance of plant

69 communities (Collins *et al.*, 2013). Ectomycorrhizal (ECM) fungi are key players in the  
70 alleviation of drought stress for their host trees, and more so as the frequency of drought  
71 events increases (Mohan *et al.*, 2014). Mycorrhizal fungi may contribute to plant water  
72 acquisition, both directly by increasing access to soil water (Allen, 2007), and indirectly  
73 by providing their host plants with nitrogen and phosphorus (Smith & Read, 2008) and  
74 improving soil structure and porosity through the formation and stabilisation of soil  
75 aggregates and organic matter (Querejeta, 2017). The relationship may also be  
76 reciprocal, as the plant host may improve water access of its associated mycorrhizal  
77 fungi through hydraulic lift, especially during summer (Querejeta *et al.*, 2003; Unestam  
78 & Sun, 1995; Querejeta, 2017). In temperate or boreal ecosystems, ectomycorrhizal  
79 fungi have been found to increase in relative abundance during summer or autumn  
80 (Wallander *et al.*, 2001; Jumpponen *et al.*, 2010; Voříšková *et al.*, 2014; Santalahti *et*  
81 *al.*, 2016), probably due to a higher below-ground allocation of host sugars during the  
82 growth season (Högberg *et al.*, 2010; Žifčáková *et al.*, 2017). However, there is a lack  
83 of information relating to intra-annual patterns in fungal community composition and  
84 biomass in Mediterranean forests soils.

85 Several studies have shown that changes in climatic conditions may affect soil fungal  
86 communities, directly or indirectly (Hartmann *et al.*, 2017; Fernandez *et al.*, 2016; Solly  
87 *et al.*, 2017). Mycorrhizal species have shown contrasting responses to changes in  
88 climate, and responses are modulated by nutrient availability (Clemmensen *et al.*, 2006;  
89 Solly *et al.*, 2017) and host tree responses (Fernandez *et al.*, 2016; Hartmann *et al.*,  
90 2017). Recent studies of Mediterranean ecosystems have suggested that the biomass of  
91 some ECM species is dynamic across seasons, and that mycelial production is often  
92 halted during summer and winter (Iotti *et al.*, 2014; Castaño *et al.*, 2017; Queralt *et al.*,  
93 2017). For example, by simulating future increases in summer drought in Mediterranean  
94 areas, we have recently predicted sharp decreases in *Lactarius vinosus* soil biomass  
95 during summer but increases during winter–spring (Castaño *et al.*, 2017). Despite these  
96 findings, similar studies considering the whole soil fungal community and biomass are  
97 still lacking in Mediterranean ecosystems.

98 Non-mycorrhizal fungal species, such as litter saprotrophs and opportunistic moulds,  
99 are also affected by changes in climatic conditions and exhibit seasonal changes in  
100 community composition, generally increasing in relative abundance under colder  
101 conditions (Jumpponen *et al.*, 2010; Andreetta *et al.*, 2011; Voříšková *et al.*, 2014;  
102 Santalahti *et al.*, 2016). In addition to the influence of microclimatic parameters, these

103 fungi may also be indirectly influenced by changes in soil properties or ground cover  
104 (Vašutová *et al.*, 2016). Climate may also select for specific groups of fungal traits  
105 (Fernandez *et al.*, 2016; Treseder & Lennon, 2015). Among these traits, ECM mycelial  
106 exploration types (i.e. long-exploration, short-exploration, contact or mat-forming) have  
107 been proposed to represent several important fungal traits that affect water and nutrient  
108 acquisition (Agerer, 2001, 2006). It has been proposed that species with short- or  
109 contact-exploration types, i.e. with mycelial biomass largely concentrated to the  
110 immediate surroundings of the roots (Agerer, 2001, 2006; Deslippe *et al.*, 2011;  
111 Fernandez *et al.*, 2016), might impose a lower carbon cost on the host plant, which  
112 could be advantageous for the host plant under stressful conditions (Fernandez *et al.*,  
113 2016). By contrast, species with more extensive mycelium (i.e. medium fringe–long  
114 distance exploration types) may be more demanding in terms of host C (Agerer, 2001;  
115 Lehto & Zwiazek, 2011; Fernandez *et al.*, 2016). The limited water availability in  
116 Mediterranean forests often results in a reduction of tree growth during summer  
117 (Sardans & Peñuelas, 2013), and likely has a negative effect on ECM fungi (Shi *et al.*,  
118 2002).

119 Here we studied monthly changes in soil fungal community composition by high-  
120 throughput sequencing of amplified fungal markers (Lindahl *et al.*, 2013) across 28  
121 long-term experimental plots over the course of a year. Our main objective was to  
122 determine whether temporal fluctuations in fungal community composition correlated  
123 with intra-annual changes in soil moisture and temperature. We also investigated micro-  
124 climatic effects on soil fungal communities by studying spatial variation between plots.  
125 The results were interpreted in the context of fungal functional guilds and traits.  
126 Responses of the total biomass of soil fungi were also assessed by analysing the fungus-  
127 specific biochemical marker ergosterol (Wallander *et al.*, 2013). We hypothesised that  
128 (i) total fungal biomass in soils would be lower during drier conditions. However,  
129 mycorrhizal fungi may be more resistant to drought, because they may use water  
130 provided by their host tree. Thus, we expected (ii) that mycorrhizal fungi would  
131 increase in abundance relative to free-living species during drier conditions. Finally, we  
132 hypothesized (iii) that responses to climate across mycorrhizal species would be related  
133 to mycelial growth form (i.e. exploration types).

## 134 **Materials and Methods**

### 135 **Site selection**

136 The study site was located in the Natural Park of Poblet (Northeast Spain, 41° 21'  
137 6.4728'' E, 1° 2' 25.7496'' N), where 28 previously established 10 × 10 m long-term  
138 monitoring plots were selected for the study. This study site has been widely used as an  
139 experimental area to study the effects of climate change in Mediterranean ecosystems  
140 (Peñuelas *et al.*, 2017). The entire experiment was initiated for a long-term project,  
141 which evaluated the mushroom production in a set of plots that were randomly  
142 distributed covering a wide range of stand characteristics. Plots consisted of even-aged  
143 (60-years-old) reforested *Pinus pinaster* (Aiton) forest, with isolated *Quercus ilex* (L.)  
144 trees, sometimes forming scrub together with other understory plant species, mostly  
145 *Erica arborea* (L.), *Arbutus unedo* (L.) and *Calluna vulgaris* (L.) Hull. Herbaceous  
146 species were rare. The plots were distributed over approximately 300 ha. area across a  
147 range of different altitudes (from 594 to 1013 m above sea level) and slopes (3–23%).  
148 The soils are characterised by siliceous minerals with franc-sandy textures, pHs ranging  
149 from 6.1 to 7.3, and organic matter contents in the upper 12 cm ranging from 3.0% to  
150 10.5%. The mean annual temperature is 11.8°C and the mean annual rainfall is 667 mm,  
151 with summer droughts usually lasting for three months (July–August–September).  
152 During the study period, the summer drought lasted for three months, with 29 mm of  
153 precipitation recorded between July and September, whereas 210 mm of precipitation  
154 were recorded in late November alone. The average temperature during the study period  
155 was 12.3°C and the total precipitation was 655.9 mm.

### 156 **Soil sampling**

157 All 28 plots were sampled monthly from June 2013 until May 2014. Each month, eight  
158 soil cores (12 cm deep and 5 cm in diameter) were collected systematically in each plot,  
159 with two cores extracted from each 10-m-side of the plot. We focused primarily on  
160 mycorrhizal fungi, and given that the fungal community composition in the needle  
161 material diverges from that of the soil (Lindahl *et al.*, 2007), we discarded intact and  
162 partially decomposed needles and sampled well-decomposed organic layers and mineral  
163 soil. Soil cores were stored at 4°C for <24 h and sieved through a 3-mm mesh. Sieved  
164 soil samples were freeze-dried and pooled to obtain a single composite soil sample for  
165 each plot and month, totalling 336 samples sub-samples, which were ground to a fine  
166 powder using mortar and pestle.

### 167 **Ergosterol analyses**

168 The total fungal biomass present in the soil was estimated by quantifying the fungal-  
169 specific biomarker ergosterol. Ergosterol was extracted as described by Nylund &

170 Wallander (1992) and chromatographically analysed as described by Hagenbo *et al.*  
171 (2017). Ergosterol data were converted to fungal biomass using a conversion factor of 3  
172  $\mu\text{g}$  ergosterol  $\text{mg}^{-1}$  dry matter (Salmanowicz & Nylund, 1988), and a correction factor  
173 (1.62) was applied to compensate for unextracted ergosterol (Montgomery *et al.*, 2000).

#### 174 **Fungal community analysis**

175 Genomic fungal DNA was extracted from 500 mg aliquots using the NucleoSpin<sup>®</sup> NSP  
176 soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol, but  
177 with 900  $\mu\text{l}$  of lysis buffer. The fungal internal transcribed spacer 2 (ITS2) region was  
178 PCR amplified in a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA, USA)  
179 using the primers gITS7 (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990), both of  
180 which were fitted with unique 8-bp tags, differing in at least three positions. The  
181 number of PCR cycles was optimised for individual samples, with most of the samples  
182 amplifying well at 21–24 cycles. The final concentrations in the 50- $\mu\text{l}$  PCR reaction  
183 mixtures were: 25 ng template, 200  $\mu\text{M}$  of each nucleotide, 2.75 mM  $\text{MgCl}_2$ , primers at  
184 200 nM and 0.025 U  $\mu\text{l}^{-1}$  polymerase (DreamTaq Green, Thermo Scientific, Waltham,  
185 MA, USA) in 1X buffer. PCR cycling conditions were as follows: 5 min at 95°C,  
186 followed by 24–30 cycles of 30 s at 95°C, 30 s at 56°C, 30 s at 72°C and a final  
187 extension step at 72°C for 7 min before storage at 4°C. Samples were amplified in  
188 triplicates with negative extraction and PCR controls. PCR products were purified using  
189 the AMPure kit (Beckman Coulter Inc. Brea, CA, USA) and quantified using a Qubit  
190 fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from  
191 each sample were pooled, and the mix was further purified using the EZNA Cycle Pure  
192 kit (Omega Bio-Tek). Quality control of purified amplicons was carried out using a  
193 BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and a 7500 DNA  
194 chip. Samples were sequenced at SciLifeLab NGI, Uppsala, Sweden on a PacBio RS II  
195 system (Pacific Biosciences, Menlo park, CA, USA) using 28 SMRT cells.

#### 196 **Bioinformatic analysis**

197 Sequences were quality filtered and clustered using the SCATA pipeline  
198 (<https://scata.mykopat.slu.se/>). Sequences with length of <200 bp were removed, after  
199 which remaining sequences were screened for primers (requiring 90% primer match)  
200 and sample tags. After the collapse of homopolymers to 3 bp, sequences were pair-wise  
201 compared using 'usearch' (Edgar, 2011). Pairwise alignments were scored using a  
202 mismatch penalty of 1, gap open penalty of 0 and a gap extension penalty of 1.  
203 Sequences were clustered into species hypotheses (Köljalg *et al.*, 2013) using single

204 linkage clustering, with a maximum distance of 1.5% to the closest neighbour required  
205 to enter clusters. Global singletons were excluded from further analyses. Sequence data  
206 are archived at NCBI's Sequence Read Archive under accession number PRJNA309233  
207 ([www.ncbi.nlm.nih.gov/sra](http://www.ncbi.nlm.nih.gov/sra)).

### 208 **Taxonomic and functional identification**

209 We assigned putative names to the 550 most abundant Species Hypotheses (SHs),  
210 which represented 93% of the total sequences (Table S1). We selected the most  
211 abundant sequence from each SH for taxonomic identification, using the massBLASter  
212 in PlutoF against the UNITE (Abarenkov *et al.*, 2010) and INSD databases. Taxonomic  
213 identities were assigned based on > 98.5% similarity with database references, or on  
214 supported monophyletic neighbour-joining clades that included reference sequences  
215 (Fig. S2). SHs were assigned to the following functional guilds: a) ectomycorrhizal, b)  
216 moulds, c) yeasts, d) black yeasts, e) litter saprotrophs, f) soil saprotrophs (saprotrophic  
217 taxa commonly found in N-rich mineral soils), g) pathogens, h) moss-associated fungi,  
218 i) root-associated ascomycetes, and j) unknown function, based on the UNITE database  
219 and DEEMY ([www.deemy.de](http://www.deemy.de)) or other published literature. Ectomycorrhizal species  
220 were assigned to exploration types according to Agerer (2001, 2006), Suz *et al.* (2014)  
221 and the dEEMY database (Agerer & Rambold, 2017), among others (Table S1).

### 222 **Climate data**

223 Volumetric soil water content and soil temperature were measured using Decagon 5 TM  
224 probes (Decagon devices Inc., Pullman, WA, USA) during the entire sampling period.  
225 Soil sensors were placed in the middle of each of the 28 plots, buried 10 cm below  
226 ground. Soil climate measurements were recorded every minute and averaged across 2-  
227 h intervals on a data logger (EM50, Decagon Devices Inc., Pullman, WA, USA). Data  
228 were downloaded and processed using the DATATRAC® III software (Pullman, WA,  
229 USA) and aggregated as monthly averages, minima and maxima.

### 230 **Data analysis**

231 Fungal community data were subjected to analyses using CANOCO version 5.0  
232 (Biometris Plant Research International, Wageningen, Netherlands) for ordinations and  
233 the 'nlme' R package for linear mixed effect models (LME; R version 3.0.2, R  
234 Development Core Team 2015).

235 Differences in soil fungal biomass were analysed using LME models after square-root  
236 transformation. Two different independent analyses were carried out to test temporal  
237 and spatial relationships between fungal biomass and soil conditions. To analyse



238 temporal variation in fungal biomass in relation to soil moisture and temperature, as  
239 well as their interaction, ‘plot identity’ was considered a random factor, whereas  
240 ‘month’ was defined as a fixed factor together with the climatic variables. To analyse  
241 spatial variation in fungal biomass in relation to soil temperature and moisture, as well  
242 as their interactions, ‘month’ was considered a random factor, whereas ‘plot identity’  
243 was defined as a fixed factor together with the climatic variables. In addition, models  
244 were tested with and without accounting for 1-week temporal autocorrelation among  
245 observations (AR1), and the most parsimonious model was selected based on the  
246 Akaike Information Criterion (AIC).

247 SH abundance data were Hellinger transformed before all multivariate analyses to  
248 account for taxa with many zeros and low count numbers. First, the relative importance  
249 of soil climatic factors, soil biochemistry and geographical distance as predictors of soil  
250 fungal community composition was evaluated by variation partitioning analyses. The  
251 first axis (PCA1) of a Principal Coordinates Analysis (PCA) of soil biochemistry  
252 parameters (Fig. S1a) was used as a biochemical index. Similarly, the first axis of a  
253 PCA of soil temperature and soil moisture was used as a soil climate index (Fig. S1b).

254 As a geographical distance index, we first calculated the principal coordinates of  
255 neighbor matrices (PCNM) spatial eigenvectors, based on UTM coordinates of the  
256 sampled plots, using Euclidean distances. We used forward selection of explanatory  
257 variables to select for significant eigenvectors. The scores of the significant spatial  
258 eigenvectors (named PCOs) for each plot were used as explanatory variables in the  
259 variation partitioning analyses, together with the soil biochemical and climate indexes.

260 Finally, in a separated variation partitioning analysis, we identified the variance  
261 explained by spatial (plot) and temporal (month) changes on the community  
262 composition, by using the “varpart” function in CANOCO.”

263 Detrended correspondence analysis (DCA) was used to obtain graphical representations  
264 of fungal community similarity between plots and months. Temporal variation in fungal  
265 community composition was related to soil moisture and temperature, as well as their  
266 interaction, by canonical correspondence analysis (CCA). Here, ‘plot identity’ was  
267 defined as a covariate, and months were randomly permuted (999 permutations),  
268 without permutation between spatial replicates within single months (i.e. effective N =  
269 12), treating ‘month’ as a repeated measure. We tested the effect of soil moisture, soil  
270 temperature and their interaction by forward selection of explanatory variables. Three  
271 independent tests were performed considering: (i) community composition at the SH

272 level, (ii) relative abundances of functional guilds and (iii) relative abundances of  
273 exploration types within the ECM community. CCA was also used to relate spatial  
274 variation in fungal community composition to soil moisture and temperature. The  
275 significance of the explanatory variables was established using Monte Carlo  
276 permutation tests (9999 permutations under the full model) without permutation of  
277 repeated observations from single plots (i.e. effective N = 28), and forward selection of  
278 explanatory variables. The same analysis was carried out using the relative proportion  
279 of each functional guild and ECM exploration type. Changes in the relative abundance  
280 of individual functional guilds and exploration types in response to variation in soil  
281 temperature and soil moisture and their interaction were assessed *post-hoc*, using LME  
282 models of square-root transformed relative proportions. In these LME models, fungal  
283 groups were defined as response variables, whereas soil moisture, soil temperature and  
284 their interaction were defined as explanatory variables (fixed terms). Both plot identity  
285 and month were included as random factors in a single analysis.

## 286 **Results**

287 The average soil fungal biomass (dry matter), as based on ergosterol measurements, was  
288  $4.9 \pm 0.1$  mg g soil<sup>-1</sup> (0.145 kg m<sup>2</sup> or 1,450 kg ha<sup>-1</sup>) and varied significantly between  
289 plots ( $F=11.06$ ,  $P<0.001$ ) and months ( $F = 5.04$ ,  $P < 0.001$ ; Fig. 1a). Fungal biomass  
290 was lowest during the summer months, especially in August (3.8 mg g soil<sup>-1</sup>), but  
291 increased during late summer–autumn to a maximum in October (5.9 mg g soil<sup>-1</sup>).  
292 Biomass decreased progressively during the winter months to another minimum in  
293 February (4.0 mg g soil<sup>-1</sup>) and increased again during the spring (Fig. 1a). At spatial  
294 scale, fungal biomass was negatively correlated with temperature ( $F=3.87$ ,  $P=0.050$ ). At  
295 temporal scale, fungal biomass was significantly correlated to the interaction between  
296 soil temperature and moisture ( $F = 5.28$ ,  $P = 0.022$ ) (Fig. 1b), with soil mycelial  
297 biomass having a negative correlation with moisture under cold conditions, a positive  
298 correlation with temperature under wet conditions, and a negative correlation with  
299 temperature under dry conditions (Fig. 1b).

300 A total of 408,788 out of 791,099 sequences (52%) passed quality filtering. Single-  
301 linkage clustering resulted in 3,063 SHs, of which 550 (93% of the high-quality  
302 sequences) were assessed for identification to species level, functional guild and  
303 exploration type (for ectomycorrhizal fungi). We obtained an average of  $27,268 \pm 3,406$   
304 reads per month and  $9,703 \pm 1,084$  reads per plot. This corresponded to  $977 \pm 353$  (SD)

305 reads per sample (min= 287 reads, max= 2,368). Overall, basidiomycota dominated the  
306 fungal community ( $56 \pm 1\%$  of the identified sequences), followed by ascomycota ( $30 \pm$   
307  $0.6\%$ ). Regarding functional guilds, mycorrhizal species were by far the most abundant  
308 ( $53 \pm 5\%$ ), followed by moulds ( $7 \pm 0.6\%$ ). Other functional groups, such as yeasts or  
309 saprotrophs, together represented 12% of the total abundance, and taxa with unknown  
310 or non-determined function accounted for  $28 \pm 0.6\%$  of the reads. The three most  
311 abundant mycorrhizal genera were *Inocybe* spp. ( $30 \pm 0.6\%$  of the mycorrhizal  
312 sequences), *Russula* spp. ( $23 \pm 0.8\%$ ) and *Tricholoma* spp. ( $8 \pm 0.4\%$ ). Among ECM  
313 exploration strategies, the short exploration type was most abundant ( $36 \pm 0.3\%$ ),  
314 followed by the contact type ( $22 \pm 0.5\%$ ), the medium-distance fringe type ( $20 \pm 0.5\%$ )  
315 and the long-distance type ( $4\% \pm 0.5\%$ ).

316 Temporal changes accounted for 3.1% of the total variance in community composition,  
317 whereas plot identity accounted for 31.8% of the total variance. At the spatial scale (plot  
318 differences), the soil climate index contributed to 4.9% of the total variance, whereas  
319 the soil biochemistry index and geographical proximity contributed to 6.4% and 5.6%  
320 of the total variance, respectively (Fig. 2a, 2b).

321 Fungal species composition varied systematically across months according to soil  
322 moisture and temperature (total monthly inertia = 0.29, adjusted explained variation =  
323 4.4%, Fig. 3a). The largest temporal differences in fungal community composition were  
324 observed between the cold and moist months (positive first-axis values, corresponding  
325 to winter months; Fig 3a) and the warm and dry months (negative first-axis values,  
326 corresponding to summer months; Fig. 3a). Forward selection indicated that soil  
327 moisture was more important in explaining temporal variation in community  
328 composition (59.7% of fitted variation,  $P = 0.002$ ) than soil temperature (40.3%,  $P =$   
329  $0.001$ ), and the interaction was not significant ( $P = 0.48$ ). Relative abundances of  
330 certain species, especially moulds (e.g. *Mortierella* and *Umbelopsis*) and yeasts (e.g.  
331 *Cryptococcus*), were higher during wetter and colder months (positive first-axis values  
332 in Fig. 4a), whereas other species, especially mycorrhizal taxa (e.g. *Russula* and  
333 *Inocybe*), were relatively more abundant during drier and warmer months (negative  
334 first-axis values in Fig. 4a).

335 Accordingly, many functional guilds correlated with soil temperature and moisture  
336 across months (pseudo- $F = 9.7$ ,  $P < 0.001$ ), accounting for 6.7 % of the total inertia  
337 (Fig. 5a). Forward selection of explanatory variables identified that soil moisture was  
338 more important (85% of fitted variation) than soil temperature (15% of fitted variation)

339 in explaining changes in guild composition across months. Exploration types of  
340 mycorrhizal species also correlated significantly with soil temperature and moisture  
341 across months (pseudo- $F = 4.7$ ,  $P = 0.004$ ), accounting for 3.3% of the total inertia  
342 (Table 1, Fig. 6a). Forward selection of explanatory variables indicated that soil  
343 temperature (87% of fitted variation) was more important than soil moisture (13% of  
344 fitted variation) in explaining the temporal variation in exploration types.  
345 Spatially, fungal species composition also varied systematically across plots according  
346 to soil moisture and temperature (Fig. 3b), with significant correlations with both soil  
347 moisture ( $F = 3.1$ ,  $P = 0.035$ ) and soil temperature ( $F = 2.7$ ,  $P = 0.008$ ), accounting for  
348 2.7% of the total inertia (Fig. 4b). Changes in the distribution of functional guilds  
349 between plots were also correlated with soil moisture ( $F = 14.2$ ,  $P = 0.006$ , Fig. 5b),  
350 accounting for 5.0% of the total inertia. Spatial variation in soil moisture, but not  
351 temperature, correlated significantly with the relative distribution of exploration types  
352 (CCA: Pseudo- $F = 7.4$ ,  $P = 0.044$ , Fig. 6b), accounting for 2.7% of the total inertia.  
353 Overall, mycorrhizal species correlated negatively with soil moisture (Table 1; Fig. 7a),  
354 whereas many free-living fungi correlated positively (Table 1; Fig 7b). The relative  
355 abundance of ECM taxa decreased from 75% of the sequences in the driest month  
356 (August) to 58% in the wettest month (February) (Fig. 7a). By contrast, the proportion  
357 of amplicons attributed to moulds (Fig. S3b), moss-associated fungi (Fig. S3c), yeasts  
358 and black yeasts correlated positively with soil moisture. The overall proportion of  
359 amplicons attributed to black yeasts (Fig. S3d) and litter saprotrophs (Fig. S3e)  
360 correlated negatively with soil temperature.  
361 Climate effects on exploration types of fungi were particularly driven by species  
362 belonging to the long-distance exploration type, which were more abundant under  
363 wetter and colder conditions (Table 1, Fig. S3f, negative first-axis in Fig. 6b), and the  
364 short-distance exploration types, which were more abundant under drier conditions  
365 (Table 1, Fig. S3g, Positive X-axis in Fig. 6b). Temperature only affected long-distance  
366 exploration types, which were more abundant under colder conditions (Fig. S3h).

## 367 **Discussion**

368 Spatial variation in fungal community composition was much larger than temporal  
369 variation. Fungal communities were affected both by soil biochemistry and soil climate,  
370 and also structured according to geographical proximity. We found that intra-annual  
371 variation in fungal biomass and fungal community composition correlated with changes

372 in soil moisture and temperature. Intra-annual changes were also found among guilds  
373 and among specific mycorrhizal exploration types. Correspondingly, spatial fungal  
374 community patterns across local plots also reflected different microclimatic features.  
375 Shifting balances between fungal functional guilds together with changes in fungal  
376 biomass suggest potential alteration in ecosystem functioning with respect to plant  
377 nutrition, soil organic matter decomposition and carbon storage (Averill *et al.*, 2014;  
378 Clemmensen *et al.*, 2015; Kvaschenko *et al.*, 2017).

379 A marked seasonality of soil fungal communities has been reported previously  
380 (Jumpponen *et al.*, 2010; Andretta *et al.*, 2011; Voříšková *et al.*, 2014), with seasonal  
381 changes being more pronounced in the topsoil layer than in deeper horizons (Andretta  
382 *et al.*, 2011; Voříšková *et al.*, 2014). Our results indicate that the soil fungal  
383 communities of Mediterranean forest ecosystems also show temporal changes within a  
384 year, although spatial variation at local scale (<3 km) is larger. Overall, soil moisture  
385 seemed to be more important than temperature in explaining variation in fungal  
386 community composition in this ecosystem. In contrast, studies of subarctic and alpine  
387 tree line ecosystems have reported that warming may cause important fungal  
388 community shifts and changes in soil fungal biomass, because higher temperatures may  
389 stimulate nutrient cycling and plant production (Clemmensen *et al.*, 2006; Solly *et al.*,  
390 2017). Here, soil fungal biomass was lower during summer than during autumn and  
391 spring, supporting previous reports from other Mediterranean forest ecosystems (Iotti *et*  
392 *al.*, 2014; Castaño *et al.*, 2017; Queralt *et al.*, 2017) and potentially reflecting  
393 differences between Mediterranean and boreal forest ecosystems.

394 We observed that functional changes in the fungal community correlated with changes  
395 in soil moisture and temperature. Drought conditions during summer were negatively  
396 correlated with soil fungal biomass in our plots, supporting our first hypothesis that  
397 summer drought would reduce soil fungal biomass. Surprisingly, however, we observed  
398 high levels of fungal biomass also under conditions of low soil moisture, such as those  
399 recorded in October, provided that soil temperatures were not too high. The increase in  
400 fungal biomass during autumn correlated with an increase in the relative abundance of  
401 ECM species. In Mediterranean forests, trees may shift their primary water source from  
402 the surface soil to groundwater during the summer (Voltas *et al.*, 2015). The capacity of  
403 deeper tree roots to access groundwater under dry conditions could help to maintain  
404 fungal symbionts via hydraulic lift (Unestam & Sun, 1995; Allen, 2007; Querejeta *et*  
405 *al.*, 2003; Querejeta, 2017). This adaptation to drought may represent an advantage for

406 root-associated symbionts versus free-living fungi (i.e. saprotrophs, moulds, yeasts and  
407 moss-associated fungi). Our results support this hypothesis, with a greater relative  
408 abundance of mycorrhizal fungi relative to free-living fungi during the summer period,  
409 as well as a higher relative proportion of mycorrhizal fungi in drier plots.

410 In other ecosystems, such as boreal or temperate forests, increasing relative abundance  
411 of ECM species has also been observed during the late growing season (Jumpponen *et*  
412 *al.*, 2010; Voříšková *et al.*, 2014; Santalahti *et al.*, 2016), concurrent with increases in  
413 total fungal biomass in soils (Wallander *et al.*, 2001; Nilsson *et al.*, 2007; Högberg *et*  
414 *al.*, 2010). Thus, the observed increase in fungal biomass during autumn (September–  
415 October) along with a higher relative representation of ECM species also supports the  
416 theory that the increasing allocation of carbon from the tree host to the roots during the  
417 autumn enhances the growth of mycorrhizal fungi (Wallander *et al.*, 2001; Högberg &  
418 Högberg, 2002; Högberg *et al.*, 2010). Potentially, there may be a link between host  
419 supply of C from photosynthesis, and water transport from roots to soil mycelium  
420 (Unestam & Sun, 1995).

421 We also observed a decreasing trend in fungal biomass during the winter months  
422 (November–February), together with a decreasing relative representation of mycorrhizal  
423 species but an increased relative abundance of saprotrophs, including yeasts  
424 (*Cryptococcus*, *Rhodotorula*), litter saprotrophs (*Mycena*) and moulds (*Mortierella*).  
425 Mycorrhizal fungi may decline during winter, when carbon allocation from the host is  
426 reduced (Jumpponen *et al.*, 2010; Voříšková *et al.*, 2014). Wetter and colder conditions  
427 during late autumn and winter may favour free-living fungi, such as moulds (Hartmann  
428 *et al.*, 2017; Castaño *et al.*, 2016; Santalahti *et al.*, 2016). Mould species can use a  
429 variety of carbohydrates, such as cellulose, pectin and starch (Thormann *et al.*, 2001)  
430 and they likely contribute to the turnover of dead mycorrhizal mycelium (Lindahl *et al.*,  
431 2010; Jumpponen *et al.*, 2010).

432 The mycelial architecture of ectomycorrhizal fungal species has been used to  
433 differentiate species traits (Agerer, 2001, 2006) with implications for nutrient and water  
434 mobilisation, as well as for the demand for host carbon. In this study, mycorrhizal  
435 species with long-distance exploration types of mycorrhiza were less abundant under  
436 drier conditions, whereas short-distance and contact species increased. This pattern was  
437 observed temporally but also spatially across plots with varying micro-climatic  
438 conditions. From a fungal perspective, drought conditions imply a moisture gradient  
439 away from the roots, with the non-suberized root tips of deeply rooted trees constituting

440 “moisture hot spots” in the otherwise dry surface soil. This soil moisture gradient away  
441 from the roots could be caused by hydraulic lift, promoting more translocated water  
442 near the roots than in the bulk soil (Querejeta *et al.*, 2003). This situation may favour  
443 fungi that focus their biomass to the interior and surface of tree roots, as well as the  
444 closer rhizosphere, over both non-symbiotic fungi and ECM fungi with extensive  
445 extramatrical mycelial proliferation. Thus, when soil becomes extremely dry, it is likely  
446 that fungi that mainly reside close to roots, such as short-distance exploration types or  
447 other low biomass ascomycetes typically found in xeric environments (Smith *et al.*,  
448 2007), have a comparative advantage over fungi with a large proportion of their  
449 mycelium in the drier bulk soil (e.g. long-distance exploration types). In addition,  
450 association with fungi with more extensive production of extraradical mycelium may  
451 imply a higher C demand on the host (Agerer, 2001, 2006) and reduce tree fitness. In  
452 contrast, ‘low biomass’ species of the contact or short-distance exploration types  
453 (Agerer, 2001, 2006; Deslippe *et al.*, 2011; Fernandez *et al.*, 2016), as well as specific  
454 stress-resistant mycorrhizal ascomycetes (Gordon & Gehring, 2011), could be less  
455 demanding in terms of host C and may, thus, be favoured under drought or other  
456 stresses.

457 It should be mentioned that long-distance exploration types were represented by only 8  
458 OTUs, all from the order Boletales, and most within the *Suillus/Rhizopogon* clade. It  
459 seems plausible that their typically extensive proliferation away from roots was  
460 disadvantageous in particularly dry soils. However, it is also possible that other traits of  
461 these species made them particularly sensitive to drought, and caution should be taken  
462 when extrapolating their negative response to other taxa within the long-distance  
463 exploration type.

464 Here, we found that below-ground fungal biomass and community composition  
465 changed in relation to intra-annual temporal and spatial climatic variation, with  
466 potential functional implications. Variable climate effects on different mycorrhizal  
467 species suggest that species may be selected or disfavoured under a climate change  
468 scenario depending on their ecological traits. Free-living fungi and mycorrhizal species  
469 with extensive mycelia could be negatively affected under increasing periods of  
470 drought. Under drought stress conditions, mycorrhizal species with mycelia  
471 concentrated more tightly around the roots may be favoured due to better water  
472 availability and lower C demand. Warmer and drier summers will most likely affect soil  
473 fungal biomass negatively, with potential to decrease saprotrophic activity. Further

474 research is needed to predict how shifts in dominance between fungal guilds and ECM  
475 exploration types in drier and warmer scenarios may affect host tree performance,  
476 drought resistance and nutrient cycling in Mediterranean forests ecosystems.

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## 490 **Author Contributions**

491 C. C. conducted soil sampling, community analysis and the statistical analysis of the  
492 data. B. D. L. supervised the community analysis and contributed to statistical analyses  
493 and data interpretation. J. G. A. contributed to statistical analyses and guided the main  
494 structure of the manuscript. A. H. contributed with ergosterol analyses and data  
495 interpretation. Javier Parladé and Joan Pera contributed with the design of the  
496 experiment and participated in the community analysis. PNIN plots have been regularly  
497 maintained by J. M. A. and J.A.B., who initiated the study and contributed with the  
498 design of the experiment, soil samplings, provided the environmental data and guided  
499 the main structure of the manuscript. All the authors contributed writing the manuscript.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

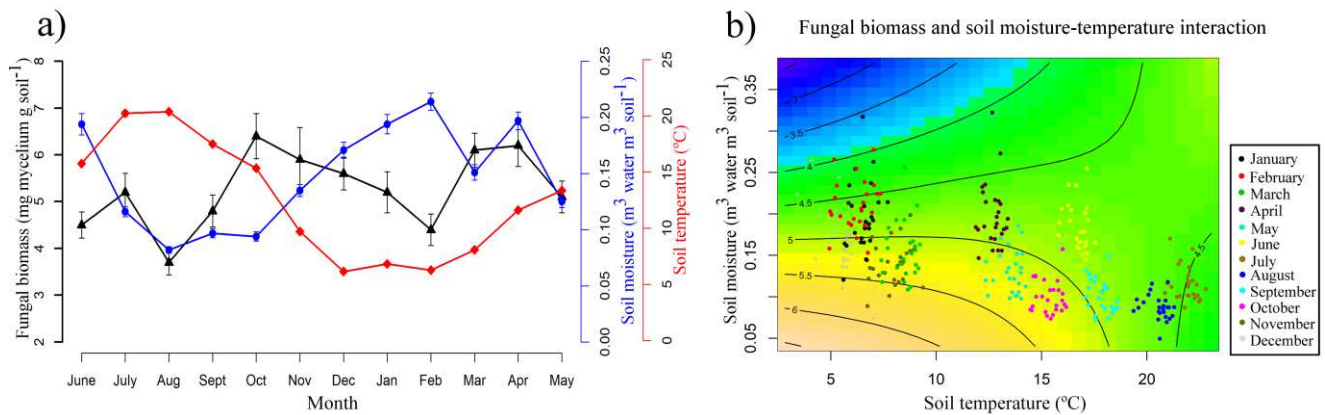
**Fig. S1** Neighbour-joining trees of representative sequences of the clusters (OTU, operational taxonomic unit) obtained and reference sequences from UNITE and INSD.

**Fig. S2** Principal component analysis of the (a) soil characteristics parameters and (b) climate-related parameters.

**Fig. S3** Mean relative abundances of relevant and significantly affected functional guilds of soil fungi and exploration types of ectomycorrhizal fungi in relation to soil moisture and soil temperature in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons. Data points represent monthly averages across 28 plots.

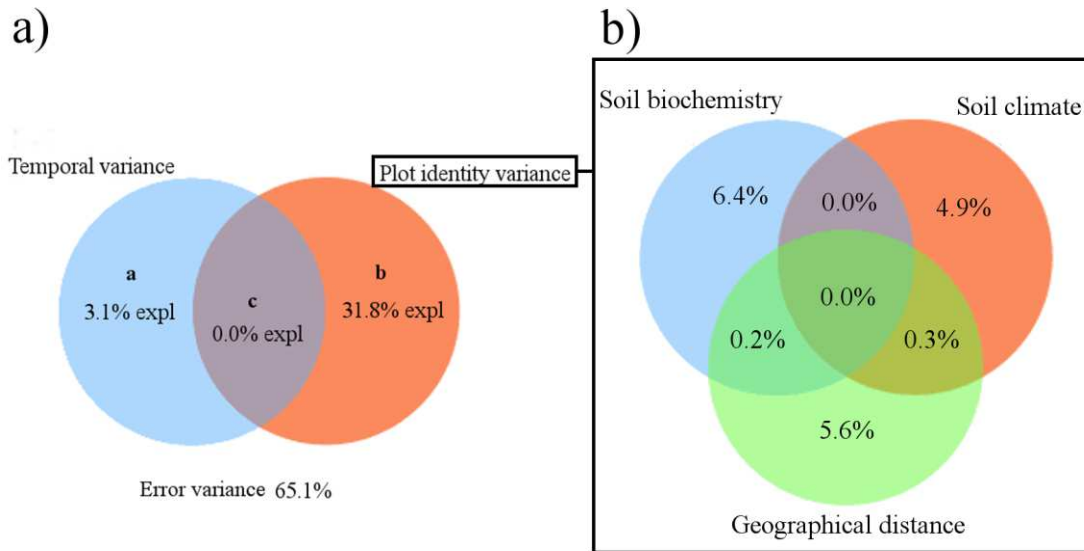
**Table S1** OTU representative identities and guild assignments

## Figures

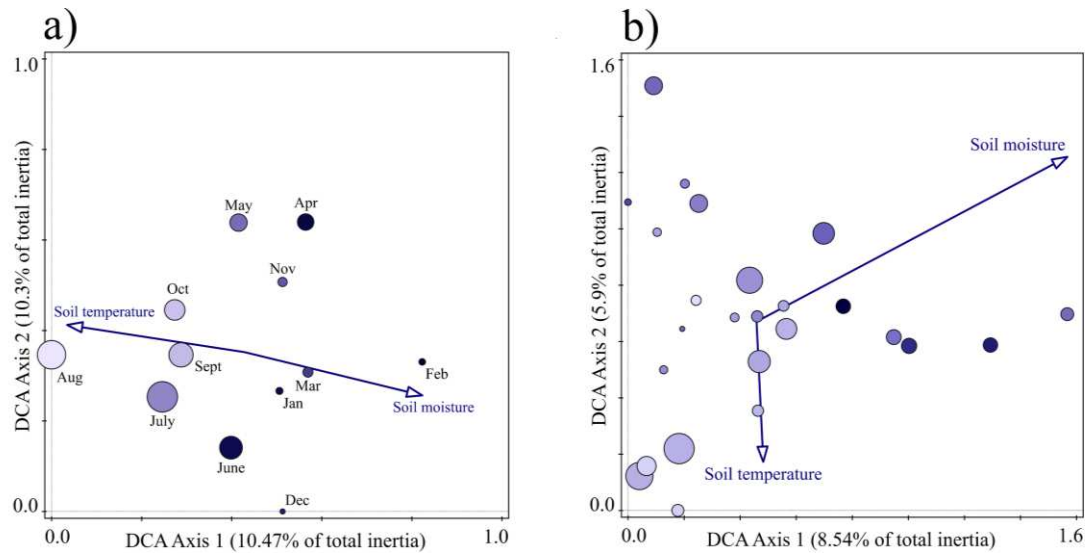


**Fig. 1** (a) Monthly averaged soil fungal biomass (mg mycelia g soil<sup>-1</sup>) over a year, based on ergosterol analysis, in a Mediterranean *Pinus pinaster* forest, and (b) correlations between soil fungal biomass and volumetric soil water content (m<sup>3</sup> water m<sup>3</sup> soil<sup>-1</sup>). Mean values ± SE are shown for the soil fungal biomass, soil moisture and soil temperature values in (a). Background colours in (b) indicate soil fungal biomass (mg mycelia g soil<sup>-1</sup>): yellow background colours represent high levels of fungal biomass (5 to 5.9 mg mycelia g soil<sup>-1</sup>), green background colours represent intermediate levels of fungal biomass (4 to 4.9 mg mycelia g soil<sup>-1</sup>), whereas blue background colours represent lower levels of fungal biomass (3 to 3.9 mg mycelia g soil<sup>-1</sup>). Coloured dots represent data points for each month.

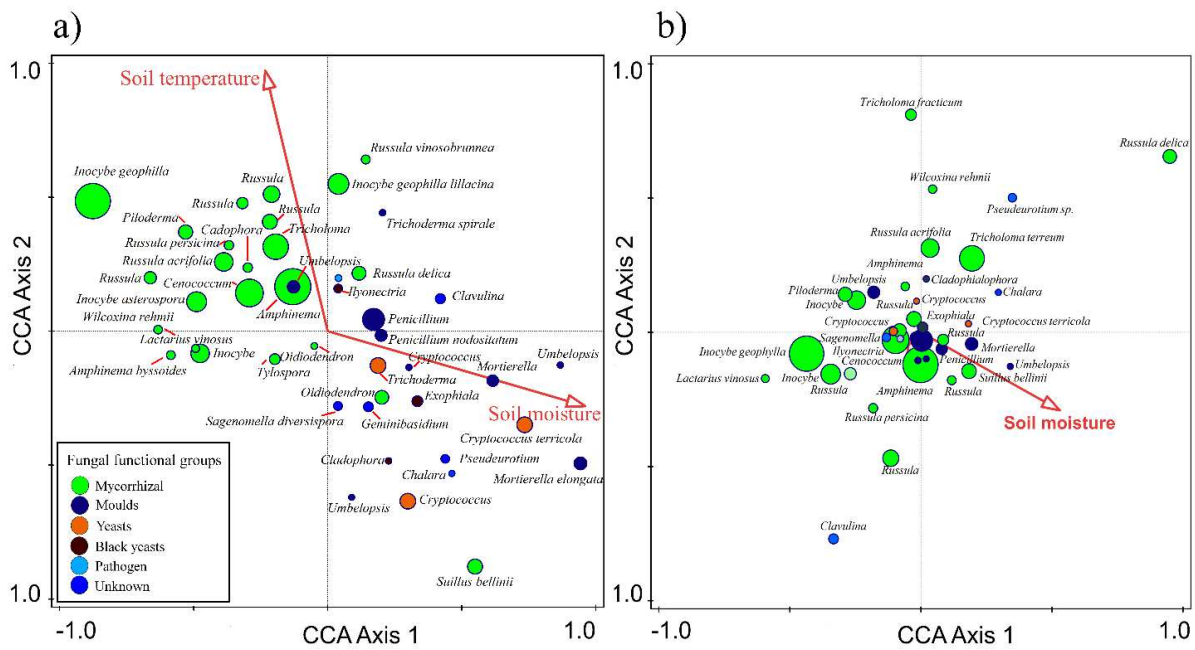




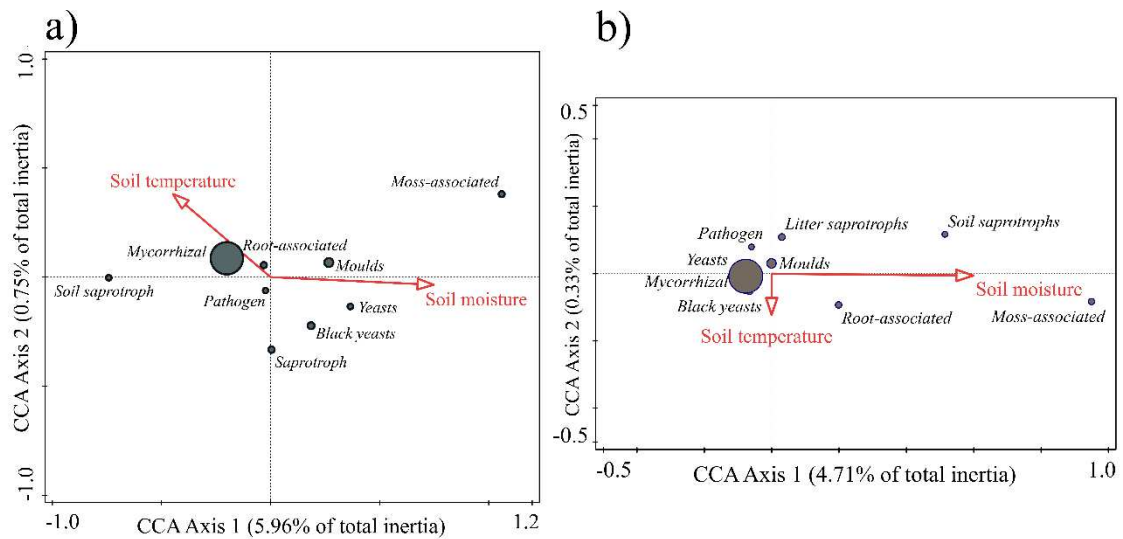
**Fig. 2** Variance partitioning analyses (a) including the plot identity effect and the temporal effects, and (b) including soil characteristics, geographical distance and soil climate on the soil fungal community. Values show the fraction of variation explained by each parameter, as well as the shared contribution of each of the parameters combination.



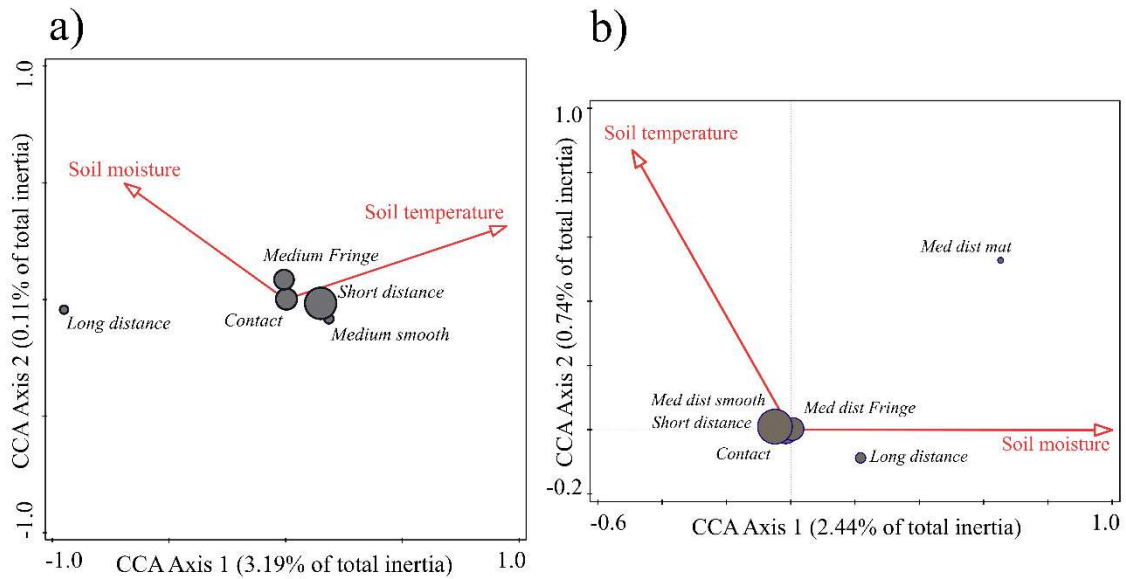
**Fig. 3** Detrended correspondence analyses (DCA) of the species level community composition of soil fungi in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons. The figures illustrate the variation in community composition (a) across months and (b) across plots. The shift from light blue to dark blue colours represents the shift in gradient from low to high soil moisture, respectively. The increasing size of the circles represents the increasing soil temperature. Soil moisture and temperature are shown as supplementary variables.



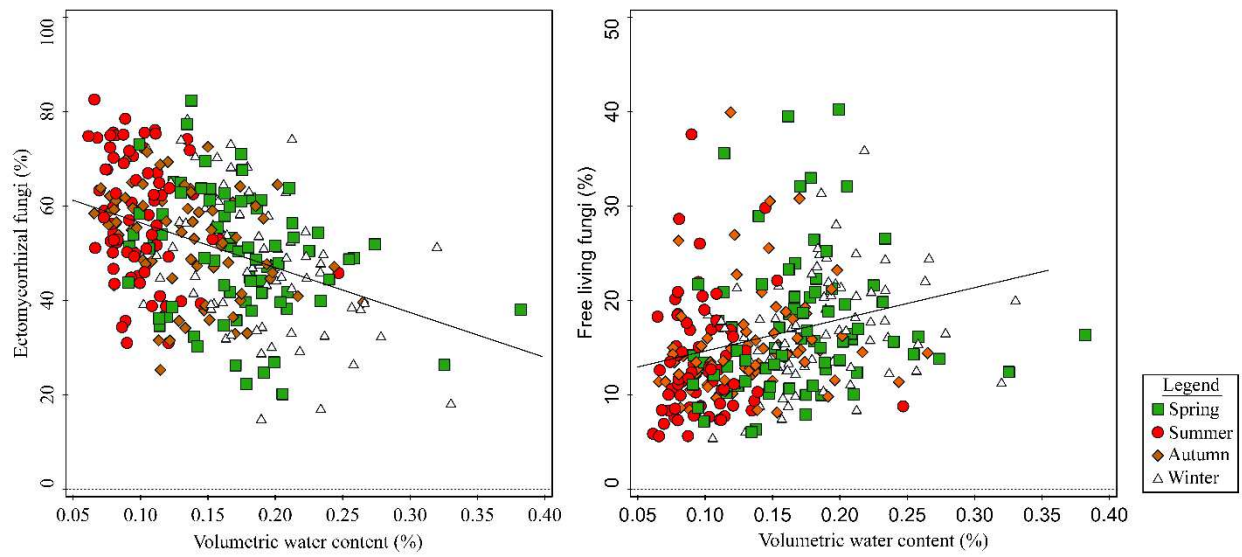
**Fig. 4** Canonical correspondence analysis (CCA) species plot showing the variation in the community composition of soil fungi in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons related to temporal changes in soil temperature and moisture. Species symbols are coloured according to functional guilds, and symbol sizes are proportional to the average relative abundance. The figure only shows the 45 most abundant species hypotheses. The analyses were based on species composition permuting (a) across months and (b) across plots.



**Fig. 5** Canonical correspondence analysis (CCA) plots considering relative proportions of functional guilds in relation to variation in soil temperature and moisture in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons. The analyses were based permuting (a) across months and (b) across plots. Symbol sizes are proportional to average relative abundances of each guild.



**Fig. 6** Canonical correspondence analyses (CCAs) plots considering relative proportions of exploration types of ectomycorrhizal species in relation to variation in soil temperature and moisture in a Mediterranean *Pinus pinaster* forest, as analysed by sequencing internal transcribed spacer 2 amplicons. The analyses were based permuting (a) across months and (b) across plots. Symbol sizes are proportional to average relative abundances of each guild. In (b), ‘*med dist*’ refers to medium distance.



**Fig. 7** Relationship between the relative abundance of (a) ectomycorrhizal fungi, (b) free-living fungi and the volumetric water content. Symbols are coloured according to season, either winter (white colour triangle), spring (light green colour square), summer (red colour circle) or autumn (brown colour diamond).

## Tables

**Table 1** Significance of linear mixed effect models of temporal and spatial correlations between relative proportions of specific functional guilds of soil fungi and exploration types of mycorrhizal fungi and climatic variables. Here, we show the soil moisture and soil temperature effects and their interaction, which were defined as fixed variables, whereas plot and month were defined as random factors. Numbers in bold indicate significant effects ( $P < 0.05$ ).

Groups	Response	Soil moisture		Soil temperature		Moisture $\times$ Temp	
		F	p	F	p	F	p
Functional guilds	Black yeast	<b>34.48</b>	<b>&lt;0.001</b>	<b>7.73</b>	<b>0.006</b>	0.85	0.356
	Mycorrhizal	<b>44.88</b>	<b>&lt;0.001</b>	1.165	0.281	0.28	0.597
	Moss associated	<b>18.20</b>	<b>&lt;0.001</b>	1.39	0.240	0.00	0.982
	Moulds	<b>100.64</b>	<b>&lt;0.001</b>	2.24	0.136	3.29	0.071
	Pathogen	0.11	0.742	0.37	0.544	0.16	0.689
	Root associated	0.26	0.610	0.29	0.589	0.08	0.781
	Saprotroph	1.04	0.307	<b>5.68</b>	<b>0.018</b>	1.12	0.291
	Soil saprotroph	10.53	0.469	0.68	0.411	0.33	0.564
	Yeast	<b>43.74</b>	<b>&lt;0.001</b>	0.62	0.430	0.12	0.724
Exploration types	Contact	0.29	0.593	0.04	0.837	<b>4.52</b>	<b>0.034</b>
	Short distance	<b>4.09</b>	<b>0.044</b>	1.09	0.297	<b>4.12</b>	<b>0.043</b>
	Long distance	<b>13.96</b>	<b>&lt;0.001</b>	<b>4.45</b>	<b>0.036</b>	<b>4.74</b>	<b>0.030</b>
	Medium Fringe	1.94	0.165	0.40	0.530	0.80	0.636
	Medium smooth	1.88	0.171	0.16	0.692	0.00	0.939
	Medium Mat	<b>4.53</b>	<b>0.034</b>	3.14	0.077	0.86	0.353

