



Variations in biomass of fungal guilds are primarily driven by factors related to soil conditions in Mediterranean *Pinus pinaster* forests

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Abstract

Soil fungi are vital for regulating ecosystem carbon balance and productivity, by driving processes related to soil carbon and nutrient cycling. The rate and capacity of fungi-mediated processes are linked to fungal biomass dynamics and identifying the drivers of fungal biomass is important for predicting ecosystem responses to environmental changes. Here, ergosterol-based fungal biomass estimates and ITS2-based fungal community composition profiles were used to assess biomass of fungal guilds. Effects of forest management (thinning), environmental factors (soil chemical properties, microclimate, weather and forest stand composition) and season were related to the fungal biomass dynamics to identify the guild-specific drivers of biomass. Biomass of most fungal guilds increased with nutrient availability (nitrogen and potassium in particular) and decreased with forest thinning, and variation in total biomass was mainly driven by variation in mycorrhizal biomass. Most fungal guilds reached a minimum in biomass during summer except for mycorrhizal and root-associated ascomycetes, which instead reached a minimum during winter. Mycorrhizal fungi and root-associated ascomycetes displayed similar spatiotemporal variability in biomass. Yeasts and moulds were the only fungi displaying strong linkages with microclimate, whereas pathogenic and moss-associated fungi largely diverged in their responses to the environmental factors. The results of our study highlight that environmental factors related to the availability of soil nutrients may have an overall stronger effect on variation in biomass of fungal guilds in Mediterranean *Pinus pinaster* forests than direct influences of microclimate, weather and forest management.

Keywords Ergosterol · Fungal biomass · Soil fungi · Mycorrhiza · Environmental drivers · Soil nutrients

Introduction

Forests are major reservoirs for terrestrial carbon (C) and are a major component of the global primary production (Pan et al. 2011). In forests, soil fungi contribute extensively to processes regulating soil C and nutrient cycles, affecting the

ecosystem C balance and productivity (Baldrian 2017). Saprotrophic fungi are major decomposers of soil organic matter (Chapin et al. 2011), symbiotic fungi contribute to the deposition of recalcitrant C (Godbold et al. 2006; Clemmensen et al. 2013) and enhance plant growth by improving nutrient uptake (Smith and Read 2008), whereas pathogenic fungi shape the vegetation composition and play an important role in maintaining biodiversity (Maron et al. 2011). Mould and yeasts are often generalist taxa with the ability to grow fast to rapidly colonise and use a wide spectrum of C sources (Algora Gallardo et al. 2021). Both mould and yeasts are contributors to soil aggregate formation, and yeasts may solubilise soil nutrients to make them more readily available for plants (Martin and Anderson 1943; Botha 2011). As microbial process and activity vary with microbial composition and biomass (Treseder et al. 2012; Graham et al. 2016; Awad et al. 2019), information on how the biomass of fungal guilds vary with environmental factors is important

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to improve our understanding of how ecosystem processes and functions may shift under climate change.

Climate change projections predict that Mediterranean ecosystems will be subjected to a 1.6 to 8.3 °C increase in mean annual temperature and a 5 to 10% decrease in precipitation by 2085 (Nogués-Bravo et al. 2008). Drought and rainfall fluctuations have strong control over fungal community composition (Sayer et al. 2017; Meisner et al. 2018), and the responses to drought vary between fungal species and guilds (Shi et al. 2002; Castaño et al. 2018). Soil physical and chemical factors interact, shaping the soil microbiome and influencing the reciprocal exchange of resources between plants and fungal communities which feedback on ecosystem functions and responses to climate change (Reynolds et al. 2003; Putten et al. 2013). Soil microclimate is coupled with weather conditions, and the soil biogeochemical cycles are strongly interlinked with soil chemical characteristics. However, the links between fungal community responses to environmental changes and ecosystem processes remain unclear (Treseder et al. 2012; Koide et al. 2014), and there is a lack of understanding of how effects of microclimate and soil chemistry relate to fungal biomass dynamics and related processes.

In Mediterranean terrestrial ecosystems, about half of the photosynthetic C is allocated belowground to roots and ectomycorrhizal fungi (Gill and Finzi 2016), which is the dominant functional guild in many Mediterranean forests (Castaño et al. 2018). Belowground C allocation is dependent on several factors, including deficiency of soil mineral nutrients associated with photosynthesis (Ericsson 1995; Gill and Finzi 2016). For example, deficiencies of potassium (K) and magnesium (Mg) typically result in a reduced belowground C allocation, whereas increased C allocation is observed under conditions of low plant-available nitrogen (N) and phosphorus (P) (Ericsson 1995). Growth of mycorrhizal mycelium has been shown to be affected by the interaction between N and P, with P starvation leading to a high mycelial production under ample N supply (Wallander and Nylund 1992; Ekblad et al. 1995). In addition to N and P availability, mycorrhizal biomass dynamics likely relate to variation in belowground C allocation (Wallander 1995; Ekblad et al. 2013; Hagenbo et al. 2017) and thus depends on host plant net C assimilation and overall performance. Water is usually the most limiting factor in Mediterranean forests, and the availability of nutrients affects the water status of plants. For example, P and K plays a central role in osmoregulation and stomatal control, hence are important for maintaining a high water-use efficiency and avoiding water stress during drought (Sardans and Peñuelas 2007, 2015; Fernández et al. 2011). Availability of Ca is important for plant water uptake by regulating water channel activity and facilitating water transport across cell membranes (White and Broadley 2003), and ample Ca supplies during

dry conditions have been shown to improve plant water status by increasing the expression of membrane proteins (aquaporins) (Galmés et al. 2007; Wang et al. 2016). Since Ca is also important for regulating cell extension, particularly in roots, a shortage of Ca may impair root growth and reduce their capacity to access deep soil layers which usually hold water reserves during the dry season (Schulze et al. 1996; Brum et al. 2017).

The contribution of environmental factors on fungal biomass and community dynamics vary with forest types and geography. In temperate forests, soil conditions (e.g. pH, soil temperature and soil moisture content) and resource availability (C in fine roots, inorganic N, soil C:N) appear to be the main drivers of the ectomycorrhizal biomass, whereas the biomass of saprotrophic fungi seems to be mainly regulated by the availability of resources and forest stand composition (Awad et al. 2019). Similarly, in alpine *Picea abies* (L.) Karts. stands, community composition of mycorrhizal and saprotrophic fungi has been shown to relate to forest stand characteristics, soil pH and soil C:N (Vašutová et al. 2017). However, the main factors regulating fungal biomass in Mediterranean forests are uncertain but opposed to temperate forests; i.e., non-mycorrhizal fungi seem to be more sensitive towards variation in microclimate (e.g. soil temperature and moisture content) than mycorrhizal fungi (Castaño et al. 2018).

In the present study, objective (1) was to identify potential drivers of fungal biomass dynamics in Mediterranean forest ecosystems. In short, we investigated the relationships between environmental variables related to soil conditions, microclimate and weather against guild-specific fungal biomass variation, which was estimated from soil ergosterol content and ITS2-based fungal community composition. The study was conducted over thinned and control *Pinus pinaster* (Aiton) forest stands to compare the relative importance between forest management and environmental factors in regulating biomass dynamics of different fungal guilds. Objective (2) was to disentangle direct linear responses of microclimate from bimodal relationships between growth and season, as we recognised that both microclimate and season may be important for regulating fungal biomass but may do so over different time scales (Alday et al. 2017; Castaño et al. 2018). This was done by studying seasonality in biomass in parallel to changes in microclimate and weather and implementing partial least squares regression analyses.

Compared to free-living fungi (e.g. yeast and moulds), mycorrhizal fungi are likely to have better access to groundwater through hydraulic lift which involves the transport of water from deeper soil layers into shallower surface layers (Querejeta et al. 2003). Based on the abundance of free-living fungi declining more than mycorrhizal fungi after dry periods (Castaño et al. 2018) and the assumption that

mycorrhizal fungi have better access to deep water reservoirs than free-living taxa, we hypothesised (1) that biomass of non-mycorrhizal fungi would primarily relate to variation in microclimate and weather. Furthermore, given the fact that mycorrhizal fungi represent a large component of the soil fungal biomass in Mediterranean forests (Castaño et al. 2018) and that the growth usually exceeds several hundred kilograms per hectare over 2–4 months (Hagenbo et al. 2021), we hypothesised that (2) the overall biomass dynamics are primarily driven by variation in mycorrhizal biomass. Furthermore, based on previous observation of excess N and P starvation resulting in an increase in mycorrhizal mycelial biomass (Wallander and Nylund 1992; Ekblad et al. 1995), we also hypothesised (3) that the mycorrhizal biomass is positively correlated with combined low extractable P and high extractable N availability. Finally, based on K and Ca being important for plant water status and thus important for maintaining photosynthesis and belowground C allocation (Ericsson 1995), we also hypothesised (4) that fungal biomass would correlate positively with the availability of K and Ca.

Materials and methods

Study sites

The study was conducted at 15 long-term monitoring plots, composed of 60-year-old *P. pinaster* stands, ranging in altitude from 594 to 1013 m above sea level, distributed

over 300 ha and located in the protected area of Poblet, in Northeastern Spain (41°21' 6.4728" E, 1°2' 25.7496" N). The stands were selected to be distant enough (at least 0.2 km apart) to be treated as independent replicates. In 13 stands, an approximate area of 40 × 40 m was subjected to thinning in July–August of 2009, resulting in paired subplots (26 in total) representing thinned and un-thinned (control) treatments. The thinning operations were conducted to assess the effects of forest management and variation in stand basal area on fungal biomass dynamics. The reduction in stand basal area by means of forest thinning varied over five different thinning intensities, representing a uniform distribution between 0 and 77% removal of the total stand basal area (Bonet et al. 2012). Felled trees were removed and *Quercus* spp. resprouts appearing punctually the following years were also removed to maintain similar conditions between thinned and un-thinned subplots. For further details see Bonet et al. (2012). The sparse understorey was mainly composed by *Quercus ilex* (L.) with occasional occurrences of *Erica arborea* (L.) and *Arbutus unedo* (L.). Herbaceous species were infrequent. The soil has a sandy loam texture and is classified as a Calcic Cambisol (IUSS Working Group WRB 2015). The mean annual temperature is 11.8 °C and the mean annual precipitation is 667 mm, with a summer drought usually occurring between July–September, lasting approximately 3 months. Variability in soil chemistry, microclimate and weather of the site are described in Table 1 and correlation between the factors is presented in Fig. S1 in Supporting Information.

Table 1 Variation soil chemistry, weather and microclimate over the study sites

Variable ¹	Mean	Median	±95% CI limit ²	Max	Min
<i>Soil chemistry</i>					
N (mg kg ⁻¹ DW)	2086	2050	10.7%	3100	1100
P (mg kg ⁻¹ DW)	7	5	22.8%	21	4
K (mg kg ⁻¹ DW)	190	176	14.9%	400	89
Mg (mg kg ⁻¹ DW)	373	378	8.2%	548	225
Na (mg kg ⁻¹ DW)	20	19	6.8%	32	15
Ca (mg kg ⁻¹ DW)	3105	2897	10.5%	5541	1814
Cation-exchange capacity (meq 100 g ⁻¹ DW)	21.9	20.5	8.8%	33.4	14.5
Organic matter content (% of DW)	5.4	5.1	13.5%	10.5	3
pH	6.7	6.8	1.6%	7.3	6
<i>Monthly weather and microclimate</i>					
Precipitation (mm)	66	36	9.5%	210	6
Air temperature (°C)	12.1	11.8	4.8%	23.1	2.8
Soil temperature (°C)	12.5	11.6	4.0%	26.1	4.6
Relative air humidity (%)	0.72	0.72	1.2%	0.95	0.28
Soil moisture content (m ³ /m ³ VWC)	0.14	0.14	3.8%	0.38	0.04

¹Dry weight and volumetric water content are indicated by DW and VWC, respectively

²Expressed as a percentage of the mean

Meteorological data

Air temperature, relative humidity of the air (RH), soil temperature and volumetric soil water content were measured in the middle of the subplots using Decagon 5™ probes (Decagon Devices Inc., Pullman, WA, USA). Measurements of air temperature and relative humidity were done at 1.3 m height and measurements of soil temperature and soil moisture content were done in the upper 10 cm of the soil. The meteorological data were based on measurements taken every minute and then averaged over 2 h and recorded in a data logger (EM50, Decagon Devices). The data was downloaded and processed using the DATATRAC III software (Pullman, WA, USA) and calculated as monthly averages. Yearly precipitation, as well as air- and soil temperature data were obtained over the same period for each plot following the DAYMET methodology (Thornton et al. 2000), as implemented in the R package ‘meteoland’ (De Cáceres et al. 2018). In short, daily precipitation and temperature were estimated for each plot by averaging the values of several Catalan and Spanish meteorological stations, applying weighting factors that depended on the geographic proximity to the target plot and correcting for differences in elevation between the station and the plot.

Soil sampling and processing

Soil was sampled from all plots in November of 2009 and monthly between June 2013 and May 2014, with a final sampling in November 2014, to facilitate comparisons between years. At each sampling, eight soil cores (12 cm long and 5 cm in diameter) were systematically sampled by collecting soil cores from each 10-m-side of the plot. Upon sampling, intact and partially decomposed needles were discarded, as fungal community composition in needle materials diverge from that of the soil (Lindahl et al. 2007). Soil samples were then stored at 4 °C for < 24 h and sieved through a 3-mm mesh, followed by freeze-drying and pooling, to obtain a single composite for each site, subplot and sampling time point. Pooled samples were then thoroughly ground using a mortar and pestle and transferred to – 20 °C storage until further analysis.

Soil chemical analyses

Organic matter contents of the pooled soil samples, representing the same subplot, were analysed according to the Walkley–Black method (Walkley and Black 1934), and extractable pools of inorganic N and P were analysed using the Kjeldahl method (Kjeldahl 1883) and the Olsen method (Olsen et al. 1954), respectively. Plant-available pools of K, Ca, Mg and Na were extracted using ammonium acetate and quantified using inductively coupled plasma optical emission

spectroscopy (ICP-OES). Cation-exchange capacity of the soils was determined by titration using ammonium acetate, as described by Chapman (1965). Soil pH was measured in a 1:2.5 soil-deionised water slurry. All soil chemistry analyses were performed by Applus Agroambiental (Applus, Madrid, Spain).

Fungal community analysis

From pooled soil samples representing the same site, fungal DNA was extracted, amplified, sequenced and bioinformatically analysed as described by Castaño et al. (2018). DNA was extracted from a subsample of 0.5 g using the NucleoSpin NSP soil kit (Macherey–Nagel, Duren, Germany), and PCR amplified in a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) using the primers gITS7 (Ihrmark et al. 2012) and ITS4 (White et al. 1990), elongated with unique identification tags. The number of PCR cycles was optimised for individual samples, amplification was done using triplicates and negative controls were carried out to detect potential contaminations of DNA or PCR products. The sample PCR products were then purified using the AMPure kit (Beckman Coulter Inc., Brea, CA, USA) and quantified using a Qubit fluorometer (Life Technologies). Equal amounts of DNA from each sample were pooled and further purified using the EZNA Cycle Pure kit (Omega Bio-Tek, Norcross, GA, USA). PCR products were checked for quality using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and sequenced using Pacific Biosciences RSII sequencing (Pacific Biosciences, Menlo Park, CA, USA) at SciLifeLab (National Genomics Infrastructure, Uppsala, Sweden) using 28 single molecule, real-time (SMRT) cells. Pacific Biosciences sequencing was selected as the sequencing method as the bias related to PCR fragment lengths is low and thus suitable for analysing ITS2 sequences which differ in length (Castaño et al. 2020). Sequences were quality filtered by the SCATA pipeline (scata.mykopat.slu.se) and clustered into species hypotheses (SHs; Kõljalg et al. 2013) using single linkage clustering. Taxonomic assignments were confirmed by using neighbour-joining phylogenetic trees (Castaño et al. 2018). The 550 most common SHs were assigned putative names and grouped into the following functional guilds: ectomycorrhizal, moulds, yeasts, saprotrophs, pathogens, moss-associated fungi and root-associated ascomycetes, based on the UNITE and DEEMY databases, as well as other published literature.

Fungal biomass quantification

Fungal biomass in samples was determined by quantifying the fungal-specific biomass marker ergosterol. In short, ergosterol was extracted as described by Nylund and Wallander (1992) and chromatographically analysed as

described by Hagenbo et al. (2017). Fungal biomass was calculated by assuming an ergosterol content of $3 \mu\text{g mg}^{-1}$ (Salmanowicz and Nylund 1988), and a correction factor (1/0.62) was applied to compensate for unextracted ergosterol (Montgomery et al. 2000). Estimates of total fungal biomass in samples were multiplied by the relative abundances of each aforementioned functional guild to obtain estimates of guild-specific fungal biomasses.

Statistical analysis

Partial least squares (PLS) regression was performed using the ‘mdatools’ package (Kucheryavskiy 2019) in R v3.5.3 (R Core Team 2013) to interpret relationships between fungal guild-specific biomasses and environmental variables of soil chemistry (nutrient availability, cation-exchange capacity, organic matter content and soil pH), microclimate (soil temperature, soil moisture content and altitude) and weather (precipitation, air temperature and relative air humidity) (Table 1), as well as forest stand density and management (stand basal area, thinned vs non-thinned plots). PLS is used similarly to multiple regression analysis but is a multivariate modelling approach in which a number of latent orthogonal factors (PLS components) are created, explaining the maximum variation in predictor- and response matrices. The latent variables are estimated consecutively and their statistical significances are evaluated by cross-validation (Wold 1978). PLS is appropriate when the explanatory variables are few and collinear (Wold et al. 2001). For all PLS models, we used tenfold cross-validation of the data, which is commonly used to evaluate model performance (McLachlan et al. 2004). Thus, the observations were randomly partitioned into 10 equal-sized subsamples and the fitting procedure was repeated for each subsample against the rest of the data, which was used as training data for the model. The average performance from 10 repetitions was then calculated to produce a single estimation of the overall model performance. All analyses were conducted using the SIMPLS algorithm, and jackknife resampling was performed during the cross-validation procedure to obtain inference statistics for the regression coefficients. The reliability of the PLS models was assessed by comparing their explained cross-validated variance (Q^2), and the importance of the individual explanatory variables was assessed using coefficient’s p values, correlation loadings and importance in projection values (VIP). The effect of random spatiotemporal variation was accounted for by including stand and sampling time (year and month) as categorical variables in all PLS regressions. Predictor and response variables were standardised by subtraction of the sample mean and then divided by the standard deviation, according to standard PLS modelling procedure.

Results

Composition of soil fungal biomass and its relationship with season

Total fungal biomass was on average 2.6 and 3.5 mg g^{-1} dry weight soil across thinned and non-thinned control plots and dominated by mycorrhizal fungi which on average contributed by 53.9% of the total fungal biomass (Fig. 1). On average, across thinned and control plots, unknown fungi (taxa with unknown function), moulds, yeasts, saprotrophs, root- and moss-associated fungi contributed by 27.0%, 7.0%, 4.4%, 3.5%, 2.0% and 1.7%, respectively, of the total fungal biomass, whereas the contribution of pathogenic fungi was < 1% (Fig. 1). Over control and thinned plots, fungal biomass varied over the season and biomass of mycorrhizal fungi and root-associated ascomycetes reached a minimum in February, whereas fungi from other guilds reached a seasonal minimum in August (Table 2).

Biomass of all fungal guilds predicted from variables of soil and microclimate

Using PLS regression to test relationships between the biomass of all fungal guilds against all the environmental factors (variables related to soil chemistry, climatic conditions and forest stand characteristics, as well as the effect of season and random site variation) resulted in a PLS model with one significant component, altogether accounting for 7.3% of the variance (R^2) in fungal biomass, and were 1.0% of the cross-validated variance (Q^2 ; model’s predictive ability) was explained (Fig. 2a). In a second PLS regression, excluding variables related to climatic conditions and forest

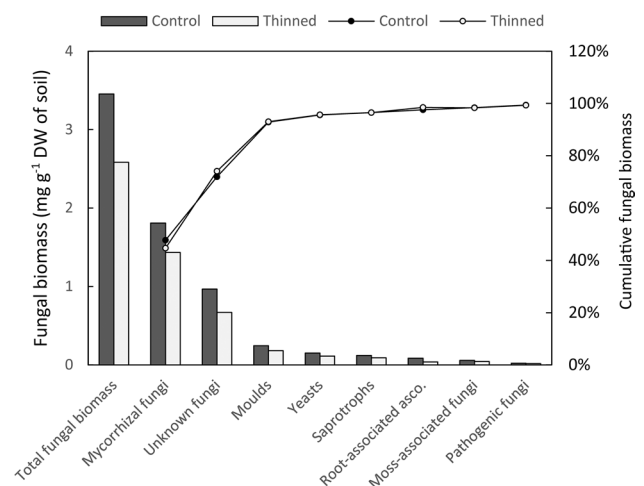


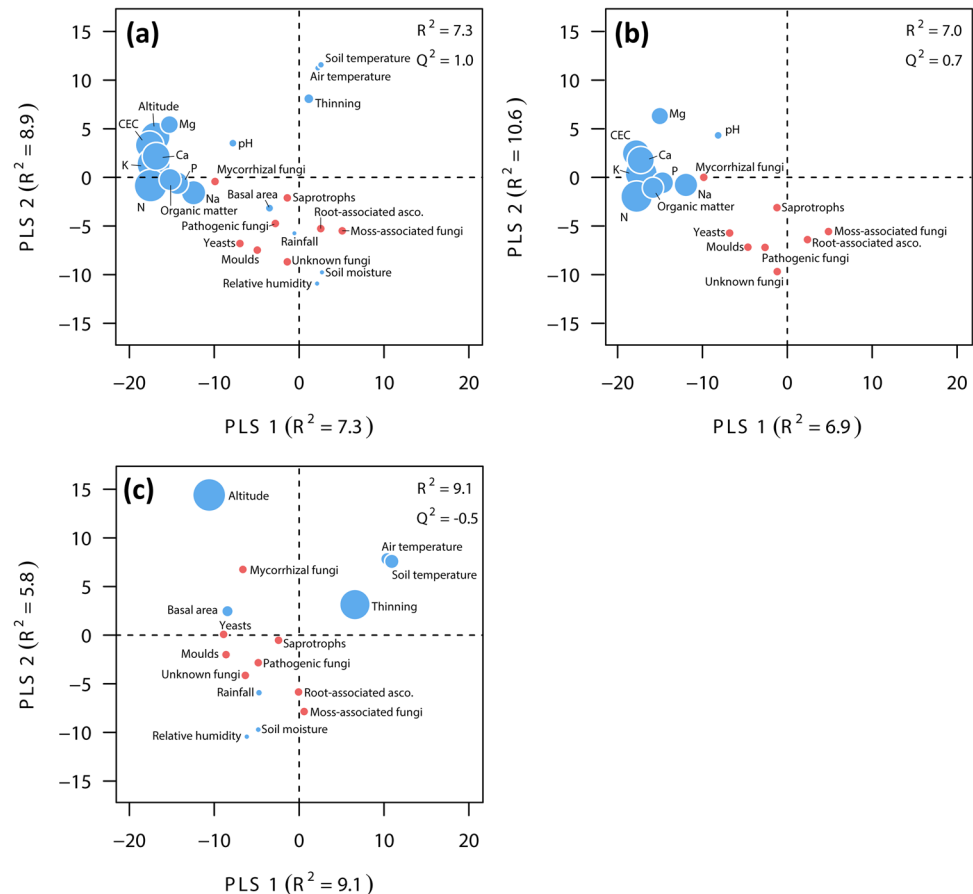
Fig. 1 Average fungal biomass and split by fungal guilds in thinned (white bars) and non-thinned control (grey bars) *Pinus pinaster* plots

Table 2 Seasonality in biomass of different fungal guilds

	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Total fungal biomass	-0.19	-0.85	0.33	0.71	-0.03	-0.32	0.41	-1.14	-	1.01	-0.48	0.85
Mycorrhizal fungi	0.21	-1.18	0.43	0.02	-0.06	-0.62	0.50	-0.58	-	0.82	-0.28	0.69
Root-associated ascomycetes	-0.52	-0.56	0.32	0.25	0.37	-0.09	0.56	-0.28	-	0.69	-0.14	0.21
Yeasts	-0.11	0.49	-0.05	1.37	-0.57	0.35	-0.36	-1.03	-	0.00	-0.82	0.86
Moulds	-0.58	0.15	-0.35	1.58	-0.44	1.32	-0.55	-1.03	-	-0.03	-0.44	0.66
Pathogenic	-0.89	-0.49	-0.64	0.62	0.31	0.19	0.11	-0.55	-	0.56	0.33	0.37
Moss-associated	0.25	-0.18	0.11	0.35	-0.17	0.14	0.58	-0.56	-	-0.02	-0.56	0.64
Saprotrophs	-0.22	-0.02	-0.05	-0.17	0.05	-0.19	0.01	-0.17	-	0.42	0.30	-0.03
Unknown	-0.54	-0.38	0.22	0.98	0.24	-0.20	0.39	-1.05	-	0.95	-0.56	0.38

Coefficient values highlighted in green- and light green colours indicate months with significantly higher biomass at $\alpha=0.01$ and 0.05 , respectively. Conversely, values highlighted in red and light red indicate months with significantly lower biomass at $\alpha=0.01$ and 0.05 . The data correspond to the coefficients of the different months, obtained from the partial least squares (PLS) regression analyses. Values of the coefficients are scaled by subtracting mean and dividing by one standard deviation followed by multiplication by ten. See Table 3 for Q^2 and R^2 values of the PLS models

Fig. 2 Partial least squares (PLS) correlation loading plots for models explaining relationships between biomass of different fungal guilds in relation to **a** all environmental variables, **b** soil properties and **c** variables related to climatic conditions. Blue and red colours of circles indicate explanatory and response variables, respectively. Values of R^2 indicate the percentage of variation explained by the models and values of Q^2 represent cross-validated explained variances and indicate how well the models predict new data. The areas of the circles are proportional to the variable importance (VIP) scores in the models and their placements indicate loading scores of the different factors. All projections (**a–c**) resulted in one significant component (PLS 1)



stand characteristics, resulted in a model ($R^2=7.0$) explaining 0.7% (Q^2) of the biomass variation of all fungal guilds (Fig. 2b). Conversely, a PLS regression excluding variables related to soil chemistry yielded a model ($R^2=9.1$) with a poor capacity ($Q^2=-0.5$) to predict variation in biomass of the fungal guilds (Fig. 2c).

Predictability was improved when the total biomass and the biomass of separate fungal guilds were tested individually against all the environmental factors (Table 3), due to loosened model constraints. PLS projection of total biomass yielded a model ($R^2=44.5$) where 32.5% of Q^2 was explained (Fig. 3a), and the first and the second component accounted for 24.2% and 20.3% of the variance in total biomass, respectively (Fig. 4a). Overall, variation in soil chemistry, climatic conditions and forest stand characteristics respectively explained 19.9%, 5.1% and 2.4% of the variance in total biomass, whereas the effect of season and random site variation accounted for 5.4% and 11.7%, respectively (Fig. 4a).

Predictability was high for PLS regressions describing biomass of mycorrhizal- and pathogenic fungi, with Q^2 values of 37.8 and 32.0, and R^2 values of 48.8 and 44.8 (Table 3, Figs. 3 and 4), and composed by two and three significant components, respectively. Variables of soil chemistry and climatic conditions respectively explained 22.0% and 6.1% of the variance in mycorrhizal fungal biomass and 18.0%

and 8.5% of the biomass variation of pathogenic fungi. The effect of season accounted for 6.7% and 8.8% of the variation in biomass of mycorrhizal and pathogenic fungi, respectively (Fig. 4). The performance was lower for PLS models describing the biomass of yeasts, unknown fungi, moulds, root-associated ascomycetes and moss-associated fungi and resulted in models with two significant components and Q^2 and R^2 values ranging from 23.3 to 11.9 and 37.0 to 30.4, respectively (Table 3). Predictability was the lowest for a PLS model describing biomass of saprotrophic fungi, with one significant component and Q^2 and R^2 values of -37.1 and 5.7, respectively (Table 3).

On average across all PLS regressions, variables related to soil chemistry, climatic conditions and forest stand characteristics respectively explained 14.6%, 5.5% and 1.5% (first and second component) of fungal guild's biomass variances, whereas the effect of season explained 5.3% of the variances (Fig. 5).

Biomass responses of fungal guilds against soil- and microclimate variables

Regression coefficients of the PLS models (Table 3) revealed that total fungal biomass decreased significantly, with thinning (standardised regression coefficient of -2.20) and

Table 3 Fungal guild biomass coefficients explaining positive and negative relationships with the environmental variables

Category	Factor	Total biomass	Mycorrhizal	Root-associated	Yeasts	Moulds	Pathogenic	Moss-associated	Saprotrophs	Unknown
Forest stand density and management	Thinning	-2.20	-1.56	-1.59	-1.45	-1.20	-0.42	-0.40	-0.16	-1.87
	Basal area	0.41	0.33	-0.66	0.22	0.77	-0.14	-0.57	0.09	0.21
Climatic conditions	Altitude	0.17	0.52	-0.90	0.13	0.14	0.30	-0.97	0.33	-0.23
	Rainfall	-0.08	-0.25	0.01	0.00	0.38	0.40	-0.29	0.18	-0.06
	Relative air humidity	0.18	-0.29	-0.46	0.08	0.16	0.02	0.67	0.09	0.48
	Air temperature	-0.34	0.02	-0.01	-0.75	-0.46	0.10	-0.34	-0.14	-0.17
	Soil temperature	-0.32	0.04	0.12	-0.89	-0.62	0.15	-0.32	-0.06	-0.12
	Soil moisture	0.04	-0.32	0.11	0.78	1.16	-0.78	0.94	-0.19	-0.26
Soil chemistry	Cation-exchange capacity	0.34	0.77	-0.47	0.00	-0.01	-0.09	-0.29	0.27	-0.33
	Organic matter content	0.34	0.17	-0.05	0.16	0.49	0.41	-0.17	0.11	0.35
	N	0.90	0.70	0.43	0.69	0.50	0.91	-0.08	0.23	0.64
	P	0.59	0.07	0.60	1.16	0.42	0.23	-0.06	0.08	0.96
	K	1.09	1.09	0.04	0.66	0.40	0.59	0.16	0.41	0.57
	Mg	-0.77	0.08	-1.26	-0.50	-0.27	-0.60	-0.72	0.03	-1.55
	Na	1.15	1.35	-0.15	0.33	0.62	-0.45	-0.63	0.35	0.33
	Ca	0.36	0.64	0.34	0.79	0.07	0.06	-0.16	0.03	-0.24
	pH	-0.58	-0.06	0.32	0.46	-0.17	-0.47	0.17	-0.27	-1.21
	R2	44.47	48.80	30.77	35.72	31.32	44.77	30.36	5.74	36.96
	Q2	32.45	37.80	13.63	23.34	18.08	31.97	11.91	-37.09	21.25
Significant components	2	2	2	2	2	3	2	1	2	
RMSE	0.919	0.564	0.078	0.068	0.119	0.023	0.104	0.148	0.420	

Coefficient values in green- and light green colours indicate positive correlations at $\alpha=0.01$ and 0.05, respectively. Conversely, values coloured red and light red indicate negative correlations at $\alpha=0.01$ and 0.05. The data correspond to the coefficients of the different environmental variables, obtained from the partial least squares (PLS) regression analyses. Values of R^2 represent the percentage of variation explained by the models and values of Q^2 represent the percentage of cross-validated explained variances and indicate how well the models predict new data. Values of the coefficients are scaled by subtracting mean and dividing by one standard deviation followed by multiplication by ten

Fig. 3 Partial least squares (PLS) correlation loading plots for models explaining relationships between environmental variables (blue circles) and variation in **a** total fungal biomass and biomass of **b** mycorrhizal fungi, **c** root-associated ascomycetes, **d** yeast fungi, **e** moulds, **f** pathogenic fungi and **g** unknown fungi in *Pinus pinaster* forests. Factors significantly explaining variation in biomass are sized according to the variable importance (VIP) scores in the models and their placements indicate their loading scores. Values of R^2 indicate the percentage of variation explained by the models and values of Q^2 represent cross-validated explained variances and indicate how well the models predict new data

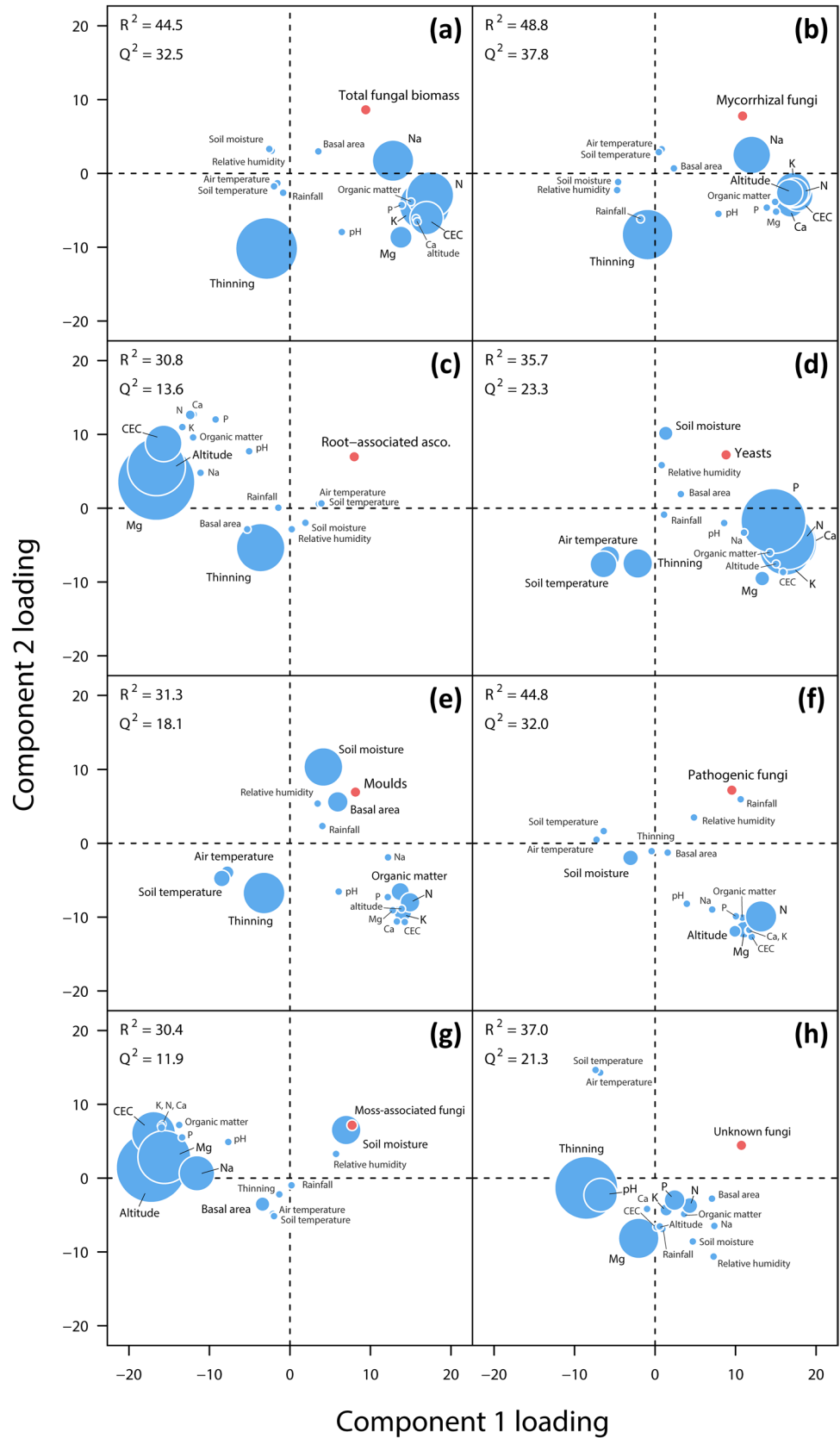
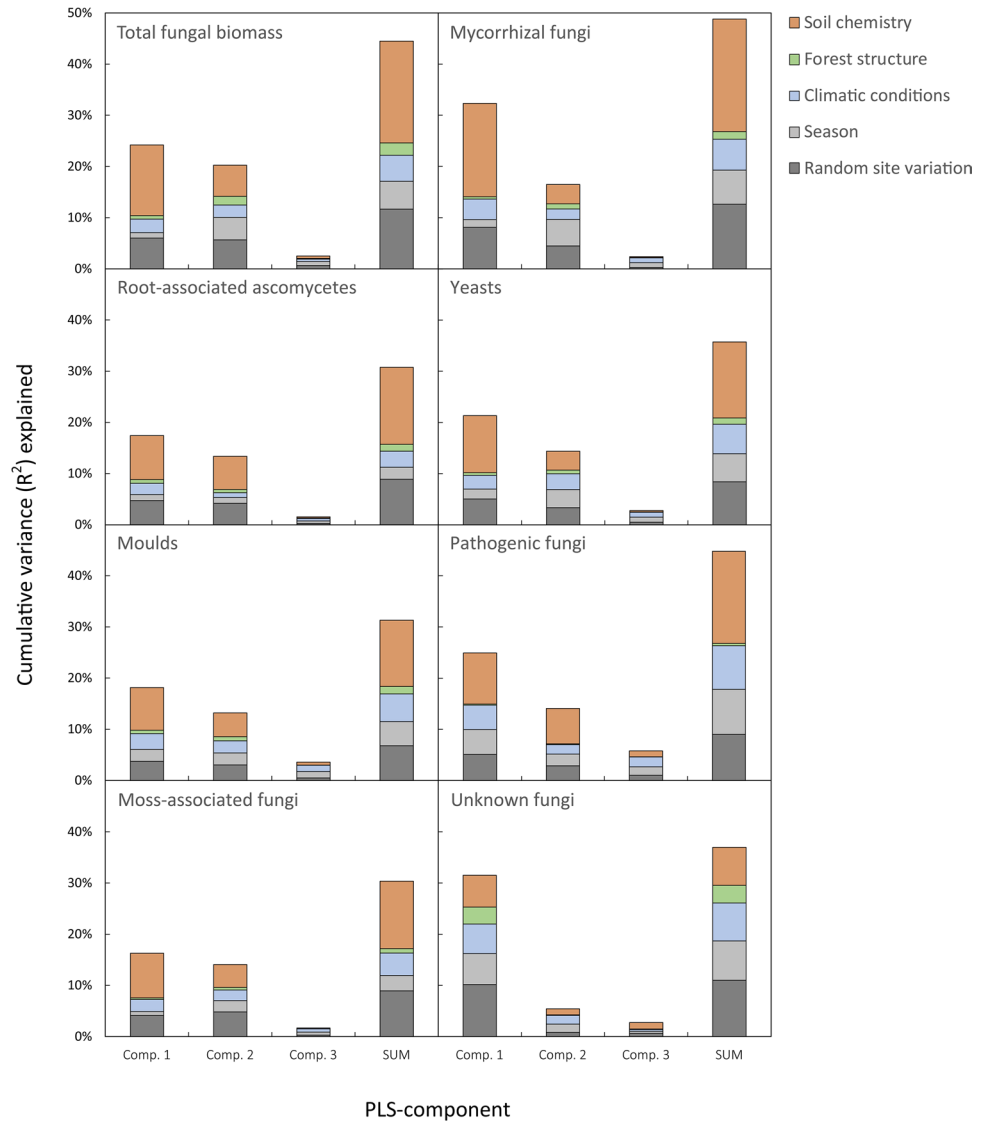


Fig. 4 Composition of partial least squares regression (PLS) components explaining seasonal variation in biomass of different fungal guilds in Mediterranean *Pinus pinaster* forest. Variables of soil chemistry (orange bars) are represented by nutrient availability, pH and soil organic matter content and variables of climatic conditions (blue bars) are represented by relative humidity, soil moisture content, air- and soil temperature, as well as precipitation



soil Mg content (−0.77), but increased significantly with Na (1.15), K (1.09), N (0.90) and cation-exchange capacity (0.34; CEC). Similarly, mycorrhizal biomass correlated negatively with thinning (−1.56) and positively with Na (1.35), K (1.09), cation-exchange capacity (0.77), N (0.70) and Ca (0.64). However, unlike the total biomass, mycorrhizal fungi correlated positively with altitude (0.52). Biomass of root-associated ascomycetes also decreased with thinning (−1.56), but opposed to mycorrhizal fungi, biomass of ascomycetes decreased with altitude (−0.90) and cation-exchange capacity (−0.47) and soil Mg content (−1.26) and displayed positive correlations with soil P- (0.60) and N content (0.43). Biomass of yeast fungi correlated negatively with thinning (−1.45) and positively with soil contents of Ca (0.79), N (0.69) and K (0.66). Biomass of yeasts also correlated negatively with air- and soil temperature (−0.75 and −0.89) and positively with soil moisture content (0.78)

and soil P content (1.16). These trends were also observed for moulds, which also decreased with thinning (−1.20) and displayed positive correlations with soil contents of N (0.50) and K (0.40). Pathogenic fungi correlated positively with soil N content (0.91) and correlated negatively with soil moisture content (−0.78), soil Mg content (−0.60) and altitude (0.30). Biomass of moss-associated fungi correlated positively with soil moisture content (0.94) and negatively with altitude (−0.97), soil contents of Mg (−0.72) and Na (−0.63), stand basal area (−0.57) and cation-exchange capacity (−0.29). Biomass of fungal taxa with unknown functions shared responses with root-associated ascomycetes and yeasts and correlated negatively with thinning (−1.87) and soil Mg content (−1.55), as well as positively with contents of P (0.96), K (0.57) and N (0.64). However, unlike the other fungal guilds, biomass of unknown fungi correlated negatively with pH (−1.21). Biomass of saprotrophic fungi

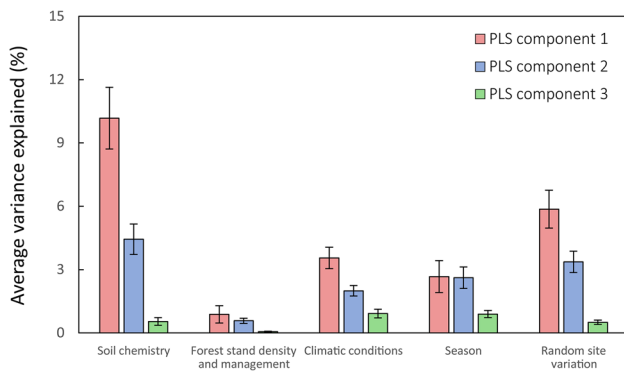


Fig. 5 Averaged explained variance and composition of the first (red), second (blue) and the third (green) partial least squares regression (PLS) component. Averaged explained variance is represented by the mean R^2 for each category of environmental factors, obtained from different PLS models describing the biomass of mycorrhizal fungi, root-associated ascomycetes, yeasts, moulds, pathogenic fungi and unknown fungi. See Fig. 4 for the composition of the PLS components of the different fungal guilds

correlated with several environmental variables. However, given the low predictive capacity of the PLS models (low Q^2), the PLS model was considered unreliable.

Discussion

In the present study, we quantified the extent to which environmental factors related to soil chemistry, microclimate, weather and forest stand density and management (basal area and effects of thinning) contribute to biomass dynamics of different fungal guilds in Mediterranean *P. pinaster* forests. Variables related to soil chemistry were the overall strongest drivers of fungal biomass, followed by the effects of season and direct influences of microclimate, weather and forest stand density and management. On average, soil chemistry variables explained 15%, while the combination of season, microclimate and weather together explained 10% of the variation in total fungal biomass. Forest density and management only explained 1.5% of the overall biomass dynamics, still thinning displayed having the single strongest influence among all the factors, contributing to a significant reduction in biomass of mycorrhizal fungi (e.g. *Inocybe*, *Russula* and *Tricholoma*), root-associated ascomycetes, yeasts (e.g. *Cryptococcus* and *Rhodotorula*) and moulds (e.g. *Mortierella* and *Umberopsis*).

Variations in fungal biomass were primarily driven by variation in soil nutrients

In disagreement with our first hypothesis, biomass of non-mycorrhizal taxa was primarily driven by factors related

to chemical- and nutrient characteristics of the soil rather than direct influences of season and microclimate. Additionally, variables related to soil conditions explained the majority (22%) of the biomass variation of mycorrhizal fungi. Thus, our results highlight that factors related to the availability of resources soil are the main drivers of fungal biomass dynamics in Mediterranean forests. This is in line with Sun et al. (2020) reporting that soil properties have an overall stronger influence on microbial biomass C than climate and ecosystem type over a global scale. Soil properties representing the strongest regulatory factor also corroborates the findings by Awad et al. (2019) reporting that variation in soil conditions is the main driver of fungal biomass dynamics in temperate European forests. The effect of season explained the second largest variation in fungal biomass, and all fungal guilds displayed seasonality in biomass. This agrees with the findings of Castaño et al. (2018), reporting that seasonal changes in climatic conditions underpin shifts in fungal community composition in Mediterranean forests. However, when disentangling the effects of season from the direct effects of precipitation, temperature and soil moisture content, we observed that those factors had a relatively small influence on the overall fungal biomass dynamics, likely because the climatic factors were largely embedded within the effect of season. However, biomass of yeasts and moulds was directly related to monthly variation in soil moisture content and temperature, which may indicate that free-living, opportunistic fungi, are more sensitive to drought and precipitation events compared to root-associated fungi (Castaño et al. 2018). Conversely, biomass of pathogenic fungi correlated negatively with soil moisture content. Unlike other fungal guilds, pathogenic fungi often exhibit a large plasticity and can thrive at water potentials well below the minimum for plant growth (Desprez-Loustau et al. 2006). Drought-induced diseases are usually caused by latent pathogenic fungi with endophytic abilities that are living inside dead parts of living trees. Thus, the negative correlation between the pathogenic biomass and soil moisture content corroborates previous findings that pathogenic fungi may interact with plant water stress to mediate drought-induced infection in trees (Desprez-Loustau et al. 2006; Allen et al. 2010). No fungal guild displayed any direct relationship with precipitation alone, likely because of a higher explanatory power of soil moisture content and season. The explanatory power was low ($Q^2 < 14$) for root-associated ascomycetes and moss-associated fungi, suggesting that some more complex mechanisms are regulating their biomass. Additionally, we were unable to explain biomass dynamics of saprotrophic fungi, likely because the upper litter layer was discarded upon sampling and only sampled well-decomposed organic layers and mineral soil.

Overall fungal biomass dynamics were primarily driven by mycorrhizal fungi

In agreement with our second hypothesis, variation in soil fungal biomass was primarily driven by mycorrhizal biomass dynamics. Thus, the factors explaining variation in mycorrhizal biomass were the overall strongest predictors of the total fungal biomass. Mycorrhizal fungi being the main driver of total fungal biomass is in line with Cheeke et al. (2021), who reported that standing fungal biomass in temperate hardwood forests in US Indiana was primarily regulated by mycorrhizal mycelial production, across a gradient in mycorrhizal association. However, in disagreement with the third hypothesis we did not observe any interaction between the levels of extractable P and N in terms of correlating with the mycorrhizal biomass, nor did we observe any significant increase in biomass with increasing soil P content. Biomass of mycorrhizal fungi relates to the availability of macronutrients, and the mycorrhizal biomass of *Pinus sylvestris* (L.) appears to peak under conditions of low P and high N (Wallander and Nylund 1992; Ekblad et al. 1995). In the present study, the fraction of easily available P was estimated using Olsen's method and soil P ranged from 4 to 21 mg kg⁻¹, which is low considering that an Olsen-P value of 20 mg kg⁻¹ has been regarded as a threshold for optimal plant growth (Li et al. 2011). Despite the apparent low P availability, it seems likely that the availability of P, relative to N, was not sufficiently low to stimulate an accumulation of mycorrhizal biomass. Furthermore, given the fact that the mycorrhizal biomass correlated positively with soil N concentration, it seems likely that the N conditions impose greater constraints on mycorrhizal growth than the apparent low P availability. However, it is difficult to establish the extent to which N and P are limited, as mycorrhizal fungi can acquire N by degrading soil organic matter (Lindahl and Tunlid 2015) and can accelerate the dissolution of mineral bound P by exuding organic acids (Schmalenberger et al. 2015).

Contrary to our findings, studies from boreal forests report decreased accumulation of mycorrhizal biomass with increased N supply (Nilsson and Wallander 2003; Kjølner et al. 2012; Bahr et al. 2013). This inconsistency between results could be related to mycorrhizal biomass accumulation not being linearly related to N availability. For example, Treseder and Allen (2002) proposed a parabolic relationship between mycorrhizal fungal growth and soil nutrient availability, with maximal mycorrhizal growth when nutrient availability constrains plant growth but not that of the mycorrhizal fungi and diminished fungal growth under low and high N availabilities. Low biomass production has also been proposed as a mechanism for limiting immobilisation in the N in fungal biomass to enable a proportionally larger host N allocation (Hagenbo et al. 2019). In addition to soil

N, the mycorrhizal biomass correlated with several factors (Na, Ca, K, CEC, thinning, altitude and season) which implies that an assembly of drivers regulates the mycorrhizal biomass dynamics in Mediterranean forests. For instance, thinning had a strong negative influence on the mycorrhizal biomass and contributed to the largest variability in biomass among all the separate factors. Although thinning increases growth of the remaining trees, stand-level productivity usually declines over the short term after thinning (Amiro et al. 2010), which may contribute to reduced C allocation to roots and associated symbionts belowground, which may impair fungal growth (Noormets et al. 2015; Collado et al. 2020).

Soil salinity may mediate variation in total- and mycorrhizal fungal biomass

In agreement with our fourth hypothesis, we observed positive correlations between the total and mycorrhizal biomass and availability of soil K and Ca, which are both involved in mechanisms mitigating drought stress in plants (Sardans and Peñuelas, 2007, 2015). Whereas Ca is important for plasma membrane stability and cell extension in roots (Thor 2019), K plays a fundamental role in maintaining osmotic homeostasis and is important for stomatal control, water conductance and root hydraulic conductivity (Sardans and Peñuelas 2015). Because restricted water access constrains photosynthesis and thus plant growth, allocation of C to belowground may decrease under severe water limitation (Staddon et al. 2004; Swaty et al. 2004), leading to reduced growth of roots and associated mycorrhizal fungi. The biomass of most fungal guilds displayed positive correlations with the extractable K content and given the importance of K in plant water uptake, it is likely that the increases in biomass are related to improved water conditions. Opposed to mycorrhizal fungi, biomass of root-associated ascomycetes displayed no correlation with K and this could be related to them being less sensitive to drought and warm conditions compared to mycorrhizal fungi and other fungal guilds (Castaño et al. 2018).

Furthermore, the positive correlation between soil Na, K, Ca and total- and mycorrhizal fungal biomass suggests that variation in salinity stress may contribute to variation in mycorrhizal biomass. In particular, the excess of Na in soil (salinity) adversely affect plant growth, by decreasing water potential in soil which impairs the uptake of water (Bolaños and Longstreth 1984). Mycorrhizal symbiosis has been proposed as a key factor for better salt tolerance of woody species by reducing Na uptake (Guerrero-Galán et al. 2019). While high salinity imposes Na toxicity and reduces fungal growth, mild-moderate Na occurrence has been reported to stimulate the growth of some mycorrhizal species (Bois et al. 2006; Matsuda et al. 2006; Zhang et al. 2008), which may contribute to the positive correlations between soil Na and mycorrhizal biomass. However, the mechanisms driving

mycorrhizal biomass responses to salinity variation are not known, and the effects of salinity on mycorrhizal-related processes need further investigation.

Conclusion

Our study shows that biomass of fungal guilds in Mediterranean *P. pinaster* forests are primarily driven by environmental factors related to soil conditions, in particular content of N and K in soil. Overall, mycorrhizal fungi and root-associated ascomycetes displayed similar biomass dynamics, yeasts and moulds were the only fungal guilds displaying strong linkages with microclimate and pathogenic and moss-associated fungi largely diverged in their responses to environmental factors. Variation in total fungal biomass was mainly driven by mycorrhizal biomass dynamics. Thus, the factors explaining variation in mycorrhizal biomass were the main contributors to total fungal biomass variation. Namely, variability in extractable soil K and Ca content had a significant influence on total and mycorrhizal biomass variation, potentially because both K and Ca being involved in mitigating drought stress in plants, thus sustaining plant growth and belowground allocation of C to roots and mycorrhizae. Additionally, soil salinity appeared to have an influence on the mycorrhizal biomass, although the mechanistic link between biomass and salinity is uncertain. All fungal guilds displayed a seasonality in biomass, whereas factors related to weather- and microclimate conditions had a limited direct influence on fungal biomass dynamics. Potentially, the lack of direct linear responses in microclimate conditions and fungal biomass dynamics is related to variation in climatic conditions being embedded within the effect of season, as well as fungal biomass dynamics operating over longer temporal scales compared to short-term meteorological conditions. While the results of our study highlight several correlations between fungal biomass dynamics and environmental factors, further research is needed to establish a mechanistic understanding of the processes driving biomass dynamics to improve our understanding of how ecosystem functions may shift with climate change.

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Availability of data and material Sequence data are archived at NCBI's Sequence Read Archive under accession number PRJNA309233 (ncbi.nlm.nih.gov/sra).

Declarations

Conflict of interest The authors declare no competing interests.

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