Ectomycorrhizal fungal network complexity determines soil multi-enzymatic activity

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Abstract

Soil functioning is intrinsically related to the structure of associated biological communities. This link is barely understood under the multi-species context of soil microbial communities, which often requires complex analytical approaches to discern into structural and functional roles of microbial taxa inhabiting the soil. To investigate these ecological properties, we characterized the assembly and soil functioning contribution of ectomycorrhizal (ECM) fungal communities through co-occurrence network analysis. Co-occurrence networks were inferred from ECM root-tips of *Cistus albidus*, *Quercus faginea* and *Q. ilex* on a regional scale, in Mediterranean mixed forests. Soil enzymatic activities related to carbon and nutrient cycling were also measured, and soil functionality outcomes related to ECM fungal network structure were evaluated from community-to-taxon level. Network complexity relied on habitat characteristics and seasonality, and it was linked to different dominant ECM fungal lineages across habitats. Soil enzymatic activities were habitat-dependent, driven by host plant identity and fungi with reduced structuring roles in the co-occurrence network (mainly within Thelephorales, Sebacinales, and Pezizales). ECM fungal co-occurrence network structure and functioning were highly context-dependent, pointing to divergent regional fungal species pools according to their niche preferences. As increased network complexity was not related to greater soil functionality, functional redundancy might be operating in Mediterranean forest soils. The revealed differentiation between structural and functional roles of ECM fungi adds new insights into the understanding of soil fungal community assembly and its functionality in ecosystems.

1. Introduction

The structure of biological communities is a key element for predicting the potential role of biodiversity in ecosystem functioning (Wagg et al., 2021). The ways through which species are assembled, e.g., *via* environmental filters, disturbances, or even by demographic stochasticity, can lead their functions within an ecosystem (Mazel et al., 2018; Wang et al., 2022). Several studies have attempted to link the structure of biological communities to their functionality, such as those relating taxonomic or phylogenetic diversity with functional traits of species or with multi-functionality indices (Delgado-Baquerizo et al., 2016; Le Bagousse-Pinguet et al., 2019; Liu et al., 2022). Some of the most widespread tools to associate community metrics with ecosystem functions are based on correlations across variables, or regression models that allow inferring ecosystem function responses through community structure predictors e.g., species richness, phylogenetic structure (Pérez-Izquierdo et al., 2017; Bastida et al., 2019; Krapu and Borsuk, 2020). However, most community structure metrics do not consider how the occurrence of species, and even their abundance, are affected by the presence/absence of other species, and to what extent this species co-occurrence might explain ecosystem functions (Wagg et al., 2019). Within this context, network analysis has emerged as a promising analytical approach to study multi-species co-occurrence and, hence, to evaluate assembly rules, niche differentiation, and expected functionality of integrated species assemblages (Mandakovic et al., 2018; Zhan et al., 2021; Goberna and Verdú, 2022).

Co-occurrence network analysis is particularly useful on data obtained from next generation sequencing at the level of Operational Taxonomic Units (OTUs), Species Hypothesis (SH) or Amplicon Sequence Variants (ASVs), as species equivalents (Barberán et al., 2012; Barroso-Bergadà et al., 2020). However, this approach is affected by biases, e.g., due to the sampling differences or intrinsic biotic and abiotic agents triggering indirect interactions that may result in spurious associations among species (Gloor et al., 2017; Barner et al., 2018; Barroso-Bergadà et al., 2021). To accurately infer species associations and overcome these constrains, several tools have been developed by applying distribution modelling or integrating correlation-based methods, such as the SParse InversE Covariance Estimation for Ecological Association Inference (SPIEC-EASI) (Kurtz et al., 2015; Morueta-Holme et al., 2016; Watts et al., 2019). These analytical frameworks have been proven useful to characterize environmental microbial communities, usually involving hyper-diverse communities with key implications for ecosystems (Pauvert et al., 2019; Wagg et al., 2019; Barroso-Bergadà et al., 2020; Luo et al., 2022).

Among soil microbial communities, ectomycorrhizal (ECM) fungi exhibit high taxonomic and functional diversity in forest ecosystems (Nguyen et al., 2020). ECM fungi mainly form symbiotic interactions in the roots of tree and shrub lineages, and are known to promote positive plant-soil feedbacks (Kadowaki et al., 2018; Bahram et al., 2020; Liang et al., 2020; Segnitz et al., 2020). In the ECM symbiosis, the plant delivers photosynthetic carbon to the fungus in exchange for nutrients that the fungus obtains through the external mycelium spreading in the soil (Smith and Read, 2008). ECM fungi are capable to scavenge nutrients from soil organic matter by producing exo-enzymes (Pritsch and Garbaye, 2011) which vary across ECM fungal lineages, promoting contrasting nutrient cycling and soil functional outcomes (Miyauchi et al., 2020; Lebreton et al., 2021; Prieto-Rubio et al., 2023). In addition, the C-enriched environment generated by the ECM symbiosis, i.e., mycorrhizosphere or hyphosphere (*sensu* van der Heijden et al., 2015), may harbour microorganisms (e.g., bacteria and saprotrophic fungi) with

enzymatic capabilities to decompose organic matter, including ECM symbiotic structures and mycelia (Azcón-Aguilar and Barea, 2015; Cheeke et al., 2017; Lebreton et al., 2021; Nguyen, 2023). Hence, as the ECM environment seems key for carbon and nutrient cycling, understanding the structure of their co-occurrence networks might bring new insights on how ECM fungal species are assembled within the communities and how they contribute to explain forest soil functioning (Barroso-Bergadà et al., 2020; Wagg et al., 2021, Martin and van der Heijden, 2024).

On the basis that the structure of ECM fungal communities is driven by habitat conditions and host plant community composition (Glassman et al., 2017; Pérez-Izquierdo et al., 2018; Wu et al., 2018), we firstly hypothesized that ECM fungal co-occurrence network structure would be dependent on host plant identity and spatial-temporal factors (habitat and seasonality). The key role of ECM fungal communities in soil functioning and soil biodiversity maintenance (Courty et al., 2010; Miyauchi et al., 2020) was at the origin of our second hypothesis: the richer and/or more complex the ECM fungal co-occurrence network, the greater soil functionality (i.e., carbon and nutrient cycling). This would be based on the associated fungal taxa with contrasting profiles of enzymatic activity leading to functional overdispersion as a result of niche segregation (Götzenberger et al., 2012; Wagg et al., 2019).

To test these hypotheses, we characterized the ECM fungal co-occurrence networks of Mediterranean mixed forests of southern Spain, at individual host plant level from three representative plant species, and measured different potential soil extracellular enzymatic activities as a proxy of carbon and nutrient mobilization. We aimed to i) infer the ECM fungal co-occurrence networks and study their structure and complexity across contrasting scales (forest, plot, host plant individual, and ECM fungal taxon); ii) determine the impact of ECM fungal co-occurrence network complexity on soil functionality; and iii) estimate the relationship between co-occurring ECM fungal taxa and the associated enzymatic activities in the soil.

2. Materials and Methods

2.1. Study sites

This study was conducted in Mediterranean mixed forests located in the natural parks of Sierras de Cazorla, Segura y Las Villas (38.29°N, -2.57°W, referred hereafter as Segura), and Monte la Sierra (37.64°N, -3.73°W, referred hereafter as Jaén), in southern Spain. These protected areas have been largely monitored by plant-plant and plant-microbe interactions to better understand topics concerning the Mediterranean forest community dynamics (from Siles et al. 2008, to Garrido et al. 2023, and Pajares-Murgó et al. 2023). With an average altitude of 1,300 m and 1,000 m in Segura and Jaén, respectively, the climate at both regions is continental Mediterranean, showing annual mean temperatures of 13-15 ºC and rainfall of 550-600 mm, and with cool wet winters and summer droughts. Soils are characterized by calcareous limestone and low nutrient retention. The canopy layer mixes *Quercus ilex* L. and *Q. faginea* Lam. with *Pinus halepensis* Mill. in Jaén, and with *Q. pyrenaica* Willd., *Pinus nigra* subsp. *salzmanii* J. F. Arnold, and *Pinus pinaster* Ait. in Segura. In both sites, the understory includes *Juniperus* spp. and tree and shrub species of Cistaceae, Oleaceae, Rosaceae, and Lamiaceae, among others (Pulgar et al., 2017; Alcántara et al., 2018). To compare ECM fungal community network-functionality outcomes across the study sites, we selected those ECM plants that were common in both study sites (i.e., *Cistus albidus* L., *Q. faginea* and *Q. ilex*).

2.2. Experimental design and sampling

Four 50 x 50 m plots were selected per each forest and samplings were carried out in autumn (2016) and spring (2017) (Alcántara et al., 2018). A total of 92 adult plant individuals were sampled, accounting 32 for *Q. ilex*, 31 for *Q. faginea*, and 29 for *C. albidus* (47 in Jaén and 45 in Segura, and 48 in autumn and 44 in spring), at least 10 m-distanced among within the given plot. After removing the litter layer, we collected three sub-samples (10 x 10 x 20 cm holes) with secondary roots tracked from the target plant and surrounding soil, and further combined into a single composite sample for each individual (Prieto-Rubio et al., 2022). Once in the lab, roots were separated from the surrounding soil, washed with tap water over 2 and 0.5 mm sieves, and all ECM root-tips collected under a Carl-Zeiss Stemi 2000 stereomicroscope (Rincón et al., 2014). Before physical-chemical analyses, the collected soil in each sample was homogenized, air-dried and 2 mm-sieved. The soil pH was measured in solutions (1:5, w:v in H_2O); the gravimetric moisture (GM) was calculated as the difference in soil weight before and after drying the samples at 105 °C for 48 h; and the soil organic matter (SOM) was determined by measuring weight loss on ignition at 400 ºC for 4 h, following the methodology in Walkley and Black (1934) (see details of soil physical-chemical analyses in Prieto-Rubio et al., 2022).

2.3. ECM fungal community and soil multi-enzymatic activity

To frame the ECM fungal community into co-occurrence networks and relate it to potential soil enzymatic activities, we used the raw data (ITS1 sequences by Illumina MiSeq) of ECM fungal communities present at root-tips, and the enzymatic activities of soils collected in the rhizosphere of *C. albidus*, *Q. ilex*, and *Q. faginea*, both at Jaén and Segura. Taxonomy of fungal amplicon sequence variants was assigned using the UNITE database v7.2 (Cole et al., 2014; Abarenkov et al., 2018) and clustered at 97% similarity into OTUs. After filtering the obtained fungal OTUs using the FUNGuild database v1.1 (Nguyen et al., 2016), the final output resulted in 449 OTUs with 6,582,941 reads associated with the ECM lifestyle. Two independent ECM abundance matrices, one per each study forest, Jaén or Segura, were used for subsequent network analyses. Further details on molecular and bioinformatics analyses are provided as Supporting information S1.

Nine potential extracellular enzymatic activities related to C, N, and P cycling in forest soils were measured: β-glucosidase, cellobiohydrolase, β-xylosidase, β-glucuronidase, and laccase related to C cycling; acid and alkaline phosphatase activities, promoting P cycling; and chitinase and leucine-aminopeptidase activities, as proxies of N cycling. Laccase was determined through a photometric assay and the rest through fluorogenic assays using a Victor microplate reader (Perkin-Elmer Life Sciences, Massachusetts, USA) (see details in Supporting information S1). A multi-enzymatic activity index (MAI) was calculated from the enzymatic activities' dataset with a Principal Component Analysis (PCA)-based procedure described in Meyer et al. (2018). Briefly, each enzymatic activity was standardized into z-scores with the *decostand* function in vegan R package (Oksanen et al., 2022). Then, a PCA was run with the *prcomp* function over the z-score dataset in *stats* R package, and PC axes were multiplied by the proportion of variation that each captured (i.e., weighted PC scores). To ensure that higher scores corresponded to higher enzymatic activities, the signs of the scores in each PC axis were inspected and the whole axis

was multiplied by -1 when necessary (re-oriented PC scores, Meyer et al., 2018; Wagg et al., 2019). The MAI index of each sample was calculated by summing its re-oriented weighted PC scores.

2.4. Co-occurrence networks in ECM fungal communities

In our study, we followed the recommendations of Goberna and Verdú (2022) concerning the use of co-occurrence concept for soil microbial communities, instead of other studies that assume direct association and/or interaction between the organisms. To infer the co-occurrence of ECM fungi within the communities, we adjusted the methodology as described by Wagg et al. (2019), which allows seeking associations among pairs of OTUs and framing them under the conditional independence concept (Kurtz et al., 2015). The OTUs abundance matrix was re-scaled by the proportion of minimum sequencing depth (39,221 sequences in Jaén and 4,076 in Segura, see Prieto-Rubio et al. 2022 for more details on sequencing yielding) and OTUs that appeared < 1% in relative abundance were discarded. We obtained two curated datasets with 47 (Jaén) and 66 (Segura) ECM fungal OTUs that were used to infer the co-occurrence matrix of each ECM fungal community, by applying the SPIEC-EASI algorithm (i.e., Sparse Inverse Covariance Estimation for Ecological Association Inference) with the function *spiec.easi* in the SpiecEasi R package (Kurtz et al., 2015). This is a robust correlation-based method for ecological network inference that differs from others in discarding spurious associations due to the lack of independence, i.e., correlations among OTUs that might be indirectly affected by other OTUs occurring in the network (Kurtz et al., 2015). The networks were constructed with the Meinshausen and Bühlmann (2006) neighbourhood selection method. The minimum lambda ratio (i.e., a scalar tuning parameter in high dimensional data) was 0.01, and 50 values of lambda for each 100 cross-validation permutations were fitted to evaluate the most parsimonious networks by using the StARS model selection criterion (Liu et al., 2010).

From the global adjacency co-occurrence matrices of each forest (*spiec.easi* objects), we extracted the presence/absence adjacency co-occurrence matrix per sample by pruning the global matrix to only include the OTUs in each sample. At the host plant individual level, we determined the OTU richness, the number of links (i.e., number of co-occurrences detected among ECM fungal taxa in each sample), and the ratio number of links / OTU richness, that was used as proxy of network complexity in each sample (Box 1). We then built weighted networks for the global adjacency matrices with the *graph.adjacency* function in igraph R package (Csardi and Nepusz, 2006), with edge weight representing the co-occurrence frequency of ECM fungal OTUs. The adjacency matrices were used to graphically represent the networks with the Gephi software v.0.9.2 (Bastian et al., 2009). Both the Fruchterman-Reingold distribution and ForceAtlas2 attraction/repulsion algorithms were used in the simulations (Fruchterman and Reingold, 1991; Jacomy et al., 2014). We obtained the network graph and calculated different network centrality metrics at the ECM fungal OTU level (degree, eigenvector, closeness, and betweenness centralities) and hub score as analogous of keystone taxa index (see Box 1). These metrics were used to measure the contribution of each OTU in structuring the co-occurrence network at site level. Network centrality metrics were performed through several algorithms in Gephi, all of them 0-1 scaled (Box 1). Further, we determined the modules (i.e., sub-communities integrating the ECM fungal co-occurrence network per site) at which OTUs belonged by using the algorithm described in Blondel et al. (2008) and implemented in Gephi.

Box 1 Ectomycorrhizal (ECM) fungal co-occurrence network parameters analyzed in this study.

OTU = Operational Taxonomic Unit.

Structural network properties	Functional network properties
OTU richness: Number of OTUs composing the co-occurrence network. Number of links: Number of co-occurrences detected among ECM fungal taxa in each sample. Network complexity: Ratio between number of links and OTU richness. De-trended network complexity for richness: calculates network complexity by discarding OTU richness intrinsic variation (Wagg et al., 2019).	Positive/negative OTU richness: Number of OTUs that explained enzymatic activities per sample. Positive/negative fungal links: number of co-occurring ECM fungal OTUs that explained enzymatic activities per sample Functional network complexity: ratio between number of positive/negative fungal links and total OTU richness. De-trended functional network complexity for richness: calculates functional network complexity by discarding OTU richness intrinsic variation (Wagg et al., 2019).
Node centrality metrics	
Eigenvector centrality: It measures the importance of a given OTU in network structuring (Mamet et al., 2019), i.e., high eigenvector centrality values are indicative of OTUs that are linked to others also relevant for network structuring, whilst low values are related to OTUs that are connected to low-impact ones in the network. Degree: It counts the number of connections per OTU (Gouveia et al., 2021), i.e., OTUs with higher degree values are more central within the network. Closeness centrality: It quantifies how influential an OTU is for the whole co-occurrence network, by calculating the average geodesic distance of a given OTU from the rest that co-occur with it (Delmas et al., 2019). Betweenness centrality: It measures the frequency of an OTU to occur within the shortest path among pairs of OTUs (Poudel et al., 2016). Hub score: Analogous index of keystone taxa, i.e., taxa that extensively contribute to community structure and function with independence of their relative abundance (Deguchi et al., 2014).	

2.5. Statistical analyses

2.5.1. ECM fungal network structure and habitat characteristics (hypothesis 1)

The response of network complexity to the factors studied was evaluated for each forest (Jaén and Segura) separately. Seasonality was fitted as fixed factor and host plant identity nested into plot as random factor by means of Linear Mixed-Effect Models (LMMs), using the lme4 R package (Bates et al., 2015). The significance of explanatory factors was tested with the functions *Anova* and *rand* from lmerTest and car R packages, respectively. The variance explained of both fixed and random factors were evaluated with the *r.squaredGLMM* function in MuMIN R package. In addition, the relationships of soil properties (log-transformed pH, SOM and GM) and network complexity parameters were assessed through Spearman correlation tests

with Bonferroni adjustment using psych and ggcorrplot R packages. The relationships between OTU-level network metrics were also evaluated by following the same correlation and adjustment tests.

175   To determine the importance of OTU abundance on network structuring (Carvalhais and Dennis, 2021), its effect on network centrality metrics (Box 1) was analysed by means of permutational ANOVA (PERMANOVA) (*adonis* function, vegan R package), using Bray-Curtis dissimilarity with 999 permutations. Besides, to evaluate whether some modules were more central than others within the ECM fungal networks, we tested centrality metrics of OTUs with the corresponding module ID by PERMANOVA.

180   **2.5.2. ECM fungal networks and soil multi-enzymatic activity (hypothesis 2)**

To detect whether the structure of ECM fungal co-occurrence networks explained soil functionality (i.e., carbon and nutrient cycling), the response of MAI was evaluated via Linear Mixed-Effect Models (LMMs) by considering OTU richness, number of links or network complexity (i.e., metrics determined at the host plant individual level), and seasonality as fixed factors. The response of de-trended network complexity, which discards the intrinsic variation derived from OTU richness, was also

185 evaluated with the same model and factors. The plant species nested in plot was considered as random factor in all LMMs.

The ECM fungal OTUs potentially affecting each soil enzymatic activity were detected by the elastic net (ENET) regularization method (Zou and Hastie, 2005; Lassalle et al., 2019). Briefly, the ENET regularization combines LASSO and Ridge penalty-based regression modelling, allowing to avoid overfitting, the correlation effects between predictors (i.e., the OTUs embedded in the co-occurrence networks), and minimizing the influence of those that lowly explain the variations in the response

190 variables (i.e., each of the soil enzymatic activities) (Zou and Hastie, 2005). As a previous step before model training, the abundance matrix of ECM fungal OTUs by host plant was standardized. Significant effects were determined by the standardized effect size $|SES| > 1.96$ (i.e., the comparison among the observed effect of the given OTU with those simulated - 999 iterations-, divided by the standard deviation of simulated ones). From the ENET results, we recorded all the OTUs predicting enzymatic activities (i.e., those that contributed to predict a given enzymatic activity) and determined the OTU

195 richness that explained enzymatic activities per host plant individual. Further, we could determine the number of co-occurring ECM fungal OTUs that supported a common enzymatic activity (i.e., positive/negative fungal links) and the functional network complexity by dividing positive/negative fungal links by total OTU richness (Box 1). In addition, as fungal network properties can be affected by the species/OTUs number, especially when diminishing (Berry and Widder, 2014), the de-trended network parameters by ECM fungal OTUs' richness allowed us to evaluate how the co-occurrence network structure yielded on multi-

200 enzymatic activity responses by discarding the variance intrinsically derived from the OTU richness. All functional network parameters recorded at each study forest (i.e., positive/negative OTU richness, positive/negative fungal links, positive/negative complexity) and their de-trended values for total OTU richness were used in linear regression models (LM) to explore their effects on MAI responses, by fitting a separate LM for each functional parameter with the *lm* function in the Stats R package.

205   **3. Results**

3.1. Structure of ECM fungal co-occurrence networks

Contrasting results in network parameters were observed between the study forests (Table 1). In Jaén, seasonality significantly affected ECM fungal network complexity i.e., lower in spring than autumn (Table 1, Fig. S1), while in Segura, this effect was detected on the OTUs richness and the number of fungal links, in both cases higher in spring than autumn. Host plant species identity affected the de-trended network complexity at the Genus level in Segura (Table 1) (*Cistus* and *Quercus* spp. respective scores of 0.16 and 0.22, $F_{1,43} = 3.88$, $p = 0.05$).

Table 1. Effects of spatial-temporal factors and host plant species on overall ectomycorrhizal fungal network properties inferred from *spiec.easi*.

		Seasonality		Host plant (nested in plot)	Variance explained	
		F-statistic	Estimate	χ^2-statistic	Fixed R^2	Random R^2
Jaén	OTU richness	0.01 ns	0.01 ± 0.10	0.01 ns	0.00	0.01
	Fungal links	3.30 .	-0.32 ± 0.17	0.46 ns	0.06	0.09
	Network complexity	**4.28 ***	**-0.06 ± 0.03**	0.37 ns	0.08	0.07
	De-trended network complexity	**4.75 ***	**-0.06 ± 0.03**	0.00 ns	0.09	0.01
Segura	OTU richness	**5.14 ***	**0.29 ± 0.13**	1.86 ns	0.09	0.15
	Fungal links	**4.22 ***	**0.45 ± 0.22**	0.23 ns	0.08	0.05
	Network complexity	2.87 .	0.05 ± 0.03	0.72 ns	0.06	0.10
	De-trended network complexity	0.26 ns	0.01 ± 0.02	**3.97 ***	0.00	0.29

Notes: Linear Mixed Models were separately performed per each study forest, Jaén and Segura. Network properties (see Box 1) were log-transformed; seasonality was fitted as fixed factor and host plant species nested into plot as random factor. The effects of fixed and random factors were determined by the F and χ^2 statistics, respectively, and the variance explained by both factor pools (R^2). Significance level: '***' $p < 0.001$, '**' $p < 0.01$, '*' $p < 0.05$, '.' $p < 0.10$, 'ns' non-significant (significant effects in bold).

When the forest was analysed as fixed factor, the ECM fungal network complexity was revealed to be significantly higher in Segura than Jaén (Fig. S2). In fact, network complexity and soil properties of each site were differentially correlated (Fig. S3). In general, soil pH was positively related to network parameters in Jaén and negatively in Segura, while higher SOM or GM values were associated with lower network complexity in Jaén and Segura, respectively. The number of modules of the inferred co-occurrence networks varied between forests (Fig. 1, Table S1). In Jaén, ECM fungal OTUs were distributed across 33 modules, the 27 % of them composed by more than one OTU, and none of them was more central than others (PERMANOVA, $F_{1,41} = 0.27$, $p = 0.67$, $R^2 = 0.01$). By contrast, ECM fungal OTUs were distributed across 27 modules in Segura, the 44 % of them with two or more OTUs, and some (e.g., modules 2, 18, 22) were more central than the rest (PERMANOVA, $F_{1,60} = 5.31$, $p = 0.02$, $R^2 = 0.08$) (Fig. 1, Table S1).
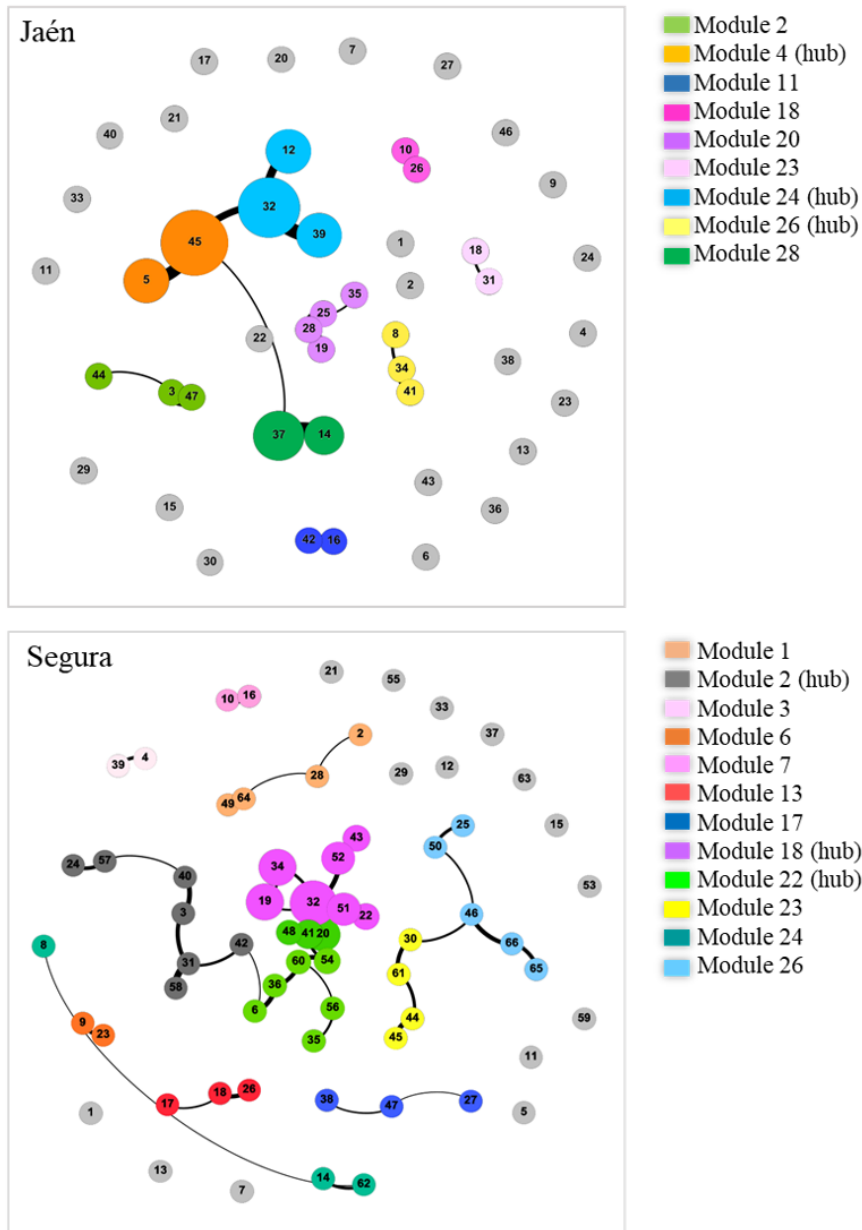
Figure 1. Co-occurrence networks of ectomycorrhizal (ECM) fungal communities, at each study forest: Jaén and Segura. Graphs are inferred through the Gephi software by using Fruchterman-Reingold and ForceAtlas algorithms. In each site network, each node number represents an ECM fungal OTU (see Table S1 for taxonomic assignment and network metrics for each forest) and the node size is related to hub score index (i.e., proxy of keystone taxa). Co-occurring OTUs are connected by edges whose width indicates the co-occurrence strength (co-exclusions were not detected among ECM fungal taxa across the study sites). Within each forest ECM fungal community, a shared node color indicates a separate module (legends) inferred by Gephi (Table S1). Nodes in light grey color indicate non-co-occurring OTUs, in each forest network.

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Negative correlations between the average abundance of ECM fungal OTUs and network centrality metrics (degree, closeness, eigenvector and betweenness centralities) were observed at both sites (Fig. 2) (PERMANOVA, $F_{1,45}$ = 4.64, p = 0.02, R^2 = 0.09 in Jaén; $F_{1,64}$ = 8.23, $p < 0.001$, R^2 = 0.11 in Segura). Correlations between centrality metrics were all positive, and overall being greater in Jaén than in Segura (Fig. 2). In addition, hub scores (i.e., proxy of keystone taxa; Box 1) were observed in specific Gephi modules, particularly in modules 4, 24, and 28 in Jaén, and 2, 18, and 22 in Segura (Fig. 1, Table S1). In Jaén, a total of seven hub OTUs were detected, most belonging to Thelephoraceae (6) (e.g., *Tomentella* (4)), and one to Russulaceae (Table S1). However, in Segura, a widespread taxonomic diversity of hub OTUs (23 in total) across different fungal families and genera e.g., *Inocybe* (7), *Tomentella* (5), *Sebacina* (4), *Tuber* (1), *Tylospora* (1), *Amphinema* (1), *Pustularia* (1), *Humaria* (1), and *Hydnum* (1) was observed (Table S1).
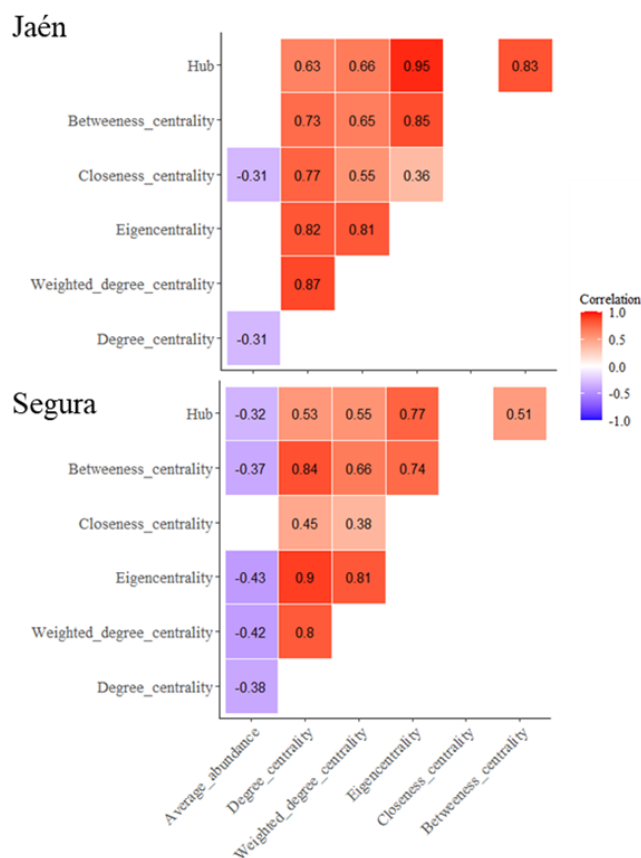
Figure 2. Correlations among average ectomycorrhizal (ECM) fungal OTU's abundance and network parameters (Box 1). Network metrics calculated for each ECM fungal OTU are inferred through the Gephi software. Correlation tests are

calculated with the Spearman method and using the Bonferroni adjustment for the dataset of each study forest, Jaén and Segura. Significant correlations ($p < 0.05$) are plotted per site, indicating positive (red) or negative (blue) correlations.

3.2. Functionality of ectomycorrhizal co-occurrence networks

255 When functional ECM fungal co-occurrence network parameters were tested as explanatory variables of the multi-enzymatic activity index MAI, a number of significant effects – positive OTU richness, de-trended positive and negative OTU richness, and functional network complexity – were observed, but only in Jaén (Table 2). In Segura, only positive OTUs were recorded, although they did not significantly affect soil MAI (Table 2); no effect of the general ECM fungal network parameters (i.e., fungal links and network complexity) on MAI was detected (Table S2).

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Table 2. Effects of functional network parameters inferred from elastic net ENET regularization models on the soil multi-enzymatic activity index (MAI) per study forest, Jaén and Segura.

	Multi-enzymatic activity index (MAI)					
	Jaén			**Segura**		
	F-statistic	Estimate	Adjusted R^2	F-statistic	Estimate	Adjusted R^2
Positive OTUs	**10.06 ****	**0.91 ± 0.29**	0.16	2.44 ns	0.41 ± 0.26	0.03
Detrended positive OTUs	**10.67 ****	**1.15 ± 0.35**	0.17	1.89 ns	0.42 ± 0.30	0.02
Negative OTUs	2.17 ns	-0.76 ± 0.51	0.02	Nr		
Detrended negative OTUs	**12.35 ****	**-2.23 ± 0.63**	0.20	Nr		
Positive fungal links	2.31 ns	0.40 ± 0.26	0.03	Nr		
Negative fungal links	Nr			Nr		
Positive network complexity	**4.15 ***	**2.56 ± 1.25**	0.06	Nr		
Negative network complexity	Nr			Nr		
De-trended positive network complexity	**3.18 .**	**2.67 ± 1.50**	0.05	Nr		
De-trended negative network complexity	Nr			Nr		

Notes: Each network metric was fitted as predictor of soil MAI in linear models, when recorded from the given forest. De-trended functional network metrics were also fitted as predictors. Significant values are highlighted in bold: '**' $p < 0.01$, '*' $p < 0.05$, '.' $p < 0.10$, 'ns' > 0.10. Nr =not recorded.

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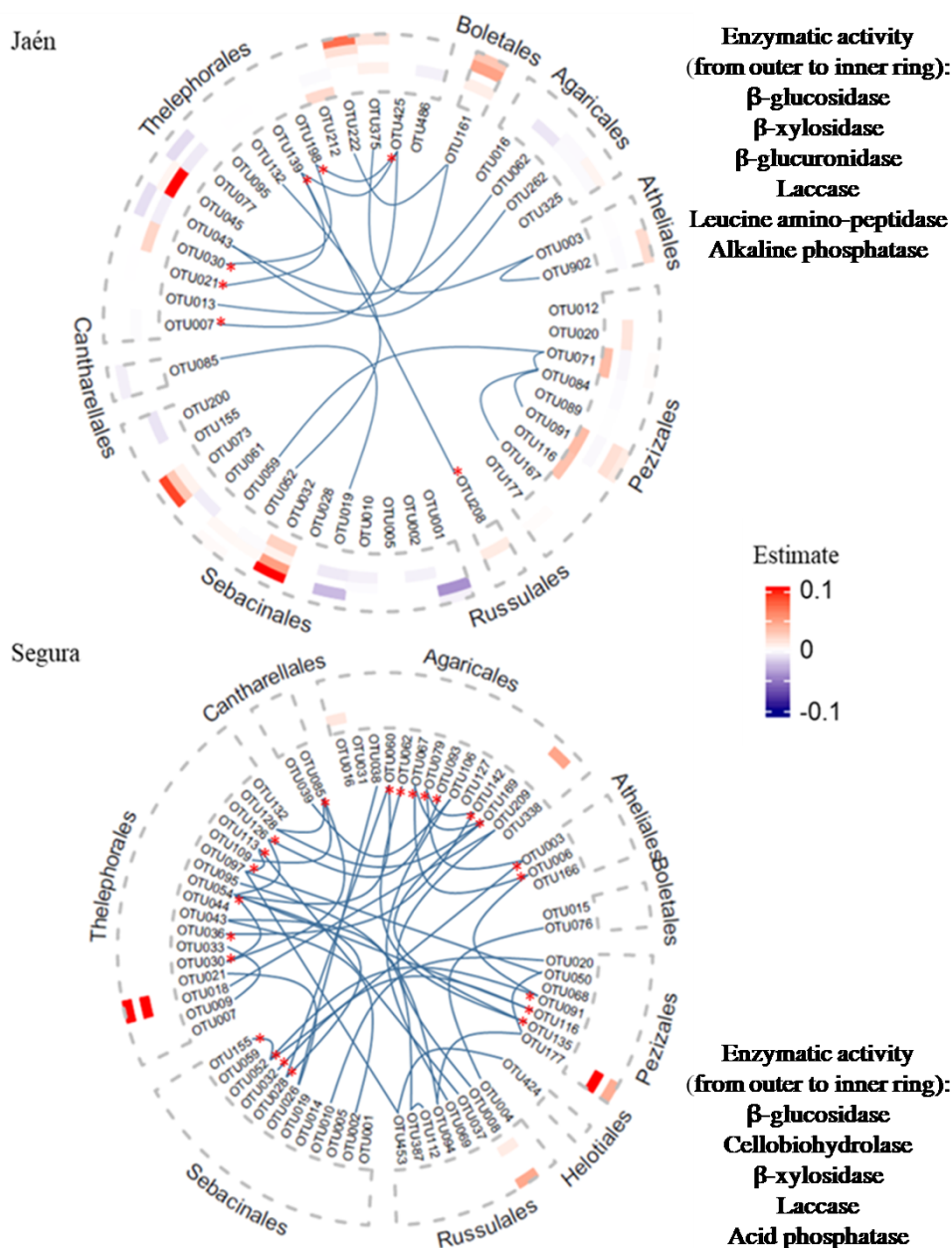
The number of OTUs that explained at least one soil enzymatic activity did greatly differ between the study forests i.e., 76.6 % and 9.1 % out of their respective total OTUs in Jaén and Segura, respectively (Fig. 3, Table S3). At both forests, ECM taxa that explained soil enzymatic activities showed low centrality scores in the co-occurrence networks (Tables S1, S3). 270 Additionally, keystone taxa (i.e., those showing hub scoring, Fig. 1) also recorded low effects on the enzymatic activities (Fig.

3). In Jaén, 36 OTUs positive- or negatively predicted soil enzymatic activities, particularly those related to C cycling (Fig. 3a, Table S3a). Most of these OTUs belonged to Thelephorales (23.40 %), Sebacinales (21.28 %), and Pezizales (12.77 %), and in many cases affecting more than one enzymatic activity, as it was also the case of the unique OTU belonging to Boletales (Fig. 3, Table S3a). Fungi within the Pezizales order showed a predominant positive effect, particularly on enzymatic activities related to C (β-glucosidase and laccase) and P (alkaline phosphatase) cycling (Fig. 3, Table S3a).

    Figure 3. Co-occurrence ectomycorrhizal (ECM) fungal networks and contribution of fungal OTUs to soil extracellular enzymatic activity, in Jaén and Segura. Co-occurring OTUs are marked with bold blue lines in the graphs, and classed by fungal orders and keystone taxa with asterisks. Heatmaps show the estimate values (positive in red and negative in blue) of ECM fungal OTUs explaining, at least, one soil enzymatic activity, by elastic net regularization ENET models. The estimate values are 0.1-scaled. We used Order as was the main resolving taxonomic category to visualize trends of ECM fungi in predicting enzymatic activities within a network context and by incorporating phylogenetic relationships. ECM fungal OTU's taxonomic assignment and estimate values are listed in Table S3.

In Segura, only 6 OTUs positively affected soil enzymatic activities (half of them either one or two enzymatic activities), particularly those related to C cycling (Fig. 3, Table S3b). The greater estimate values were detected for *Peziza succosella* and Thelephoraceae sp., which positively explained β-glucosidase and β-xylosidase activities (Fig. 3, Table S3b).

4. Discussion

ECM fungal co-occurrence networks in Mediterranean forest soils reveal a strong habitat dependency, supporting our first hypothesis. Network metrics differ between the two study forests by season, and also by the host plant identity (i.e., *Cistus* vs. *Quercus* spp.) in Segura. When investigating further into the ECM fungal co-occurring network properties, the abundance of occurring taxa appears as a key parameter for network structuring since, for a given ECM fungal OTU, centrality metrics diminish with increasing OTU abundance. The co-occurrence network structure does not have a significant effect on soil multi-enzymatic activity index MAI, but we do find relationships when functional network metrics are analysed (e.g., OTUs and their co-occurrences explaining at least one soil enzymatic activity), partially supporting our second hypothesis. Our study reveals that fungal OTUs within a co-occurrence network context may explain soil enzymatic activities (mostly those related to carbon cycling), and that major effects are directed by taxa belonging to the orders Thelephorales, Sebacinales, and Pezizales.

4.1. Ectomycorrhizal fungal networks differed between Mediterranean mixed forests

ECM fungal co-occurrence networks in the study Mediterranean mixed forests showed a strong habitat dependency. Environmental factors such as summer drought, wildfire recurrence or soil nutrient retention, have been pointed as important long-term habitat filters affecting Mediterranean forest community adaptations (Pérez-Valera et al., 2017; Pérez-Izquierdo et al., 2020; Hernandez et al., 2021). These filters could contribute to the regional changes in fungal OTU richness and network parameters observed in this study. In addition, soil properties were related to the outcomes associated with co-occurrence network complexity. Co-occurrence networks revealed differential responses to soil pH -ranging from 6.9 to 8.3 (averaging 7.7 ± 0.1) and from 4.9 to 8.2 (averaging 6.6 ± 0.2), in Jaén and Segura respectively-, suggesting local adaptation to homogeneous neutral-alkaline soils in Jaén in contrast with those more variable in Segura, which also depended on the host

plant species identity e.g., higher soil pH under *C. albidus* than *Quercus* spp. (Prieto-Rubio et al., 2023). On the basis that pH is a well-known filter for soil microbial community assembly (Glassman et al., 2017; Tripathi et al., 2018), our results suggest that neutral pH contributed to diminish network complexity showing an increased number of potential single-OTU modules (Jaén). By contrast, neutral-to-acidic conditions could imply a wider range of available niches for ECM fungi, what would explain the increasing fungal network complexity observed in Segura.

The seasonality effect on the network parameters varied at each study forest, suggesting that this effect is dependent on the habitat characteristics. This habitat-dependency of seasonal outcomes associated with nutrient availability and fungal communities in forest soils has been previously shown (Siles and Margesin, 2017; Rincón et al., 2015; Ji et al., 2021). In fact, the altitudinal variations between Jaén and Segura (~ 300 m) may imply variations in physical-chemical and SOM properties, as previously shown in other Mediterranean forest ecosystems (Gutiérrez-Girón et al., 2015; Centenaro et al., 2023). These altitudinal variations also span the limit between meso- and supra-Mediterranean bioclimatic belts (~ 1,200 m.a.s.l.) that lead changes in physiological responses of host plant communities (Rivas-Martínez, 1987), and likely also on the ECM fungal co-occurrence networks. Further studies including a broader altitudinal zonation would be needed to confirm this causal effect on Mediterranean forest soil communities.

4.2. The dominance of certain ECM fungi was key in network structuring

The richness and abundance of ECM fungal taxa were key to understand how co-occurrence networks were structured. As argued by Wagg et al. (2019), the network complexity de-trended by OTU richness discards the intrinsic variation due to OTU richness. This parameter revealed a habitat effect, that is, in the low complexity network of Jaén, de-trending network metrics helped finding fine-tuned community outcomes that would be obscured by the low OTU richness, e.g., detecting seasonal variations. By contrast, in the more complex network of Segura, those seasonal variations were not detected but unveiled a host plant effect, differing at the genus level (*Cistus* vs. *Quercus*) (as previously observed when phylogenetic turnover outcomes of the ECM fungal communities were evaluated, Prieto-Rubio et al., 2022). The network composition also varied between the studied forests, but revealed common fungal orders (Pezizales, Sebacinales, and Thelephorales) that dominated the network in Jaén (~80 % of total OTUs), whilst others (e.g., Agaricales, Boletales or Russulales) rarely occurred. In Segura, the abundance of the common fungal orders found in Jaén decreased, allowing other fungal lineages to increase their network representativeness (e.g., OTUs belonging to Agaricales and Atheliales). This contrasting pattern among fungal lineages might suggest that ECM fungal communities conserved a regional species pool with lineages likely differing by their ecological niche space, as observed in arbuscular mycorrhizal fungal communities by Davison et al. (2021).

The negative correlation found among fungal abundance with centrality metrics suggests that local dominance plays a key role on ECM fungal network structuring (Lamanna et al., 2016; Prieto-Rubio et al., 2022). This property of ECM fungi is consistent with that observed in arbuscular mycorrhizal fungal communities (Davison et al., 2022), and its extent could also be influenced by the environmental context (Wang et al., 2022). In Jaén, the edaphic environment (neutral-alkaline soils with high organic matter content and homogeneous under all host plant species, Prieto-Rubio et al., 2023) could limit niche breadth for ECM

fungi, likely leaving a more important role to dominance and explaining the observed lower community richness and network complexity (Davison et al., 2022). Whilst, in Segura, where soil properties were more differentiated across host plant species (Prieto-Rubio et al., 2022, 2023), the increasing niche breadth for ECM fungi could mitigate the role of dominance (Davison et al., 2022). This would facilitate the coexistence among more fungal taxa, hence, explaining the higher observed community richness and network complexity at this forest (Chomicki et al., 2019). Moreover, the outcomes concerning keystone taxa could also be affected by dominance, as revealing that these OTUs were not locally-abundant, confirming that other mechanisms beyond competitive exclusion, such as those related to demographic stochasticity (e.g., priority effects), could impact the co-occurrence network structure (Kennedy et al., 2009; Debray et al., 2022). Further studies de-coupling the role of niche processes from that of stochasticity on interaction/co-occurrence networks will bring novel understandings of fungal community assembly.

4.3. ECM fungal network structure predicted enzymatic activities in forest soils

Contrary to our expectations, the overall network properties did barely explain soil multi-enzymatic activity index (MAI). But when functional network properties inferred from ENET regularization were tested, we found that an increase in the number of ECM fungal OTUs positively related to any soil enzymatic activity, leading to soil MAI positive values. These patterns were mainly observed in Jaén, where a greater number of ECM fungal taxa explained any soil enzymatic activity compared with those in Segura. The effects of ECM fungal networks on enzymatic activities configuring soil MAI were also visualized through the co-occurrence networks. Across the study forests, different ECM fungal lineages could be linked with the enzymatic activities, as previously observed in soil microbial communities from different ecotones (Trivedi et al., 2016; Banerjee et al., 2018a). The number of ECM fungal OTUs explaining soil enzymatic activities substantially differed between the study forests, with a greater number of OTUs explaining enzymatic activities in Jaén than in Segura. In accordance with microbial networks in grasslands (Wagg et al., 2019; Lou et al., 2022), we observed that highly complex ECM fungal networks presented fewer taxa explaining enzymatic activity rates in forest soils, i.e., as if increasing network complexity would lead into functional redundancy, likely explained by the functional complementarity among OTUs conforming the network (Wang et al., 2021). This result supporting the ideas of functional redundancy (i.e., enzymatic activity conservation across ECM fungi, Baldrian and Kohout, 2017) fits well into the holobiont theory (Zobel et al., 2024). Indeed, from the holobiont perspective, this redundancy ensures that essential functions carried out by microbiota are maintained even if one or more species are lost or disrupted, providing resilience against environmental changes, disturbances, or the loss of specific microbial species (Vandenkoornhuyse et al. 2015). Alternatively, other soil microbial groups could be contributing to P and N mobilization into a much greater extent than ECM in these forest soils (Ward et al., 2021; Xun et al., 2021).

Furthermore, we found that the effects of ECM fungal taxa on soil enzymatic activities varied across ECM fungal lineages. In Jaén, positive or negative effects on soil enzymatic activities related to C mobilization were shown in taxa belonging to Agaricales, Atheliales, Cantharellales, Pezizales, and Russulales. These ECM fungal lineages are known to conserve exo-

enzyme pools related to C cycling (Miyauchi et al., 2020). We could also unveil that OTUs belonging to Pezizales mainly drove P mobilization, whilst taxa contributing to explain N cycling were few but phylogenetically widespread across ECM fungal lineages. Our results also show that keystone ECM fungal taxa were not major determinants of soil enzymatic activities, especially in Segura, where few effects were detected despite of the higher richness and network complexity. This suggests

380 that keystone ECM fungal taxa play community assembly-related roles rather than those associated with biogeochemical functions, as initially assumed for these microbial groups in soil networks (Banerjee et al., 2018b). These findings evidence the need to integrate in further studies other microbial guilds (e.g., saprotrophic bacteria and fungi) on co-occurrence networks to deepen into the soil community attributes that explain carbon and nutrient mobilization.

5. Conclusions

385 This study shows the importance to integrate co-occurrence network analyses to disentangle the ectomycorrhizal fungal community structure and function. We conclude that the ECM co-occurrence network outcomes are highly dependent on the habitat characteristics, and that increased network complexity could imply functional redundancy in forest soils. The abundance of ECM fungal taxa is key to understand the network structure, supporting the potential role of dominance on the ECM fungal community assembly in Mediterranean forests. At this co-occurrence network context, we found that several

390 ECM fungal lineages explained soil functioning through enzymatic activities. In addition, keystone ECM fungal taxa were not necessarily those that profoundly explain soil enzymatic activities, showing a main influence on the network structuring. Incorporating other soil microbial guilds (e.g., saprotrophs, pathogens, arbuscular mycorrhizas) in co-occurrence network analyses will help to accurately discern across structural and biogeochemical roles within forest soil communities.

395 **Data/code availability**

Datasets and R codes are available upon request.

Author contributions

JMA, CA and AR designed the research; JPR, JLG, JMA, CA and AR performed sampling; JPR did laboratory work; JPR,
400 ALG and AR analysed data and JPR wrote the manuscript. ALG and AR contributed equally. All authors revised the manuscript.

Competing interest

The authors declare that they have no conflict of interest.

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