Supplementary Information

Title: Ectomycorrhizal fungal network complexity determines soil multi-enzymatic activity

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Supporting information S1

S1.1. Molecular analysis and bioinformatics

The molecular analysis and bioinformatics are detailed in Prieto-Rubio et al. (2022). Briefly, ~ 30 mg of ECM root tips per sample were mixed with polyvinylpyrrolidone (PVPP). The genomic DNA was extracted with the Invisorb®DNA Plant HTS 96 Kit/C kit (Invitek GmbH, Berlin, Germany). The fungal ITS1 region was amplified with the primer set ITS1F-ITS2 (Gardes and Bruns, 1993) under the following PCR conditions: 3 min at 94°C, 30 cycles of 1 min at 94°C, 30 s at 53°C and 45 s at 72°C, and a final step of 10 min at 72°C (Pérez-Izquierdo et al., 2020). Fungal amplicons were sequenced by Illumina MiSeq at the Fundación Parque Científico de Madrid, Spain.

The R open-source DADA2 pipeline v1.16 (Callahan et al., 2016; R Core Team 2020) was used to process the raw sequences by using default parameters. The taxonomic assignment for each obtained Amplicon Sequence Variant (ASV) was carried out by using the RDP algorithm and the UNITE database v7.2 (Cole et al., 2014; Abarenkov et al., 2018). A 97 % similarity-clustering was carried out with vsearch in mothur (Schloss et al. 2009; Rognes et al. 2016) to obtain Operational Taxonomic Units (OTUs). The OTU x sample matrix was curated using the LULU algorithm (Frøslev et al., 2017; Pauvert et al., 2019b).

The obtained fungal OTUs were classified into guilds (i.e., ECM fungi, arbuscular mycorrhizal fungi, pathogens, saprotrophs) by using the FUNGuild database v1.0 (Nguyen et al. 2016) and bibliographic support (Agerer, 2006; Tedersoo and Smith 2013; Tedersoo et al., 2014). The final output resulted in 983 fungal OTUs and 6,847,682 reads, from which a subset of 449 OTUs and 6,582,941 reads were associated with the ECM lifestyle. An ECM fungal abundance matrix per study site, Jaén and Segura, was prepared for further data analyses.

S1.2. Measurement of soil enzymatic activities

We determined nine potential extracellular enzymatic activities related to C, N and P cycling in forest soils (German et al., 2011; Pierre-Emmanuel et al., 2016; Pérez-Izquierdo et al., 2018; Stock et al., 2019) in soil samples: β-glucosidase (EC 3.2.1.3), cellobiohydrolase (EC 3.2.1.91), β-xylosidase (EC 3.2.1.37) and β-glucuronidase (EC 3.2.1.31), related to C cycling; acid and alkaline phosphatase (EC 3.1.3.2) activities, promoting P cycling; and chitinase (EC 3.2.1.14) and leucine-aminopeptidase (EC 3.4.11.1) activities, as proxies of N cycling. In addition, laccase activity (EC 1.10.3.2), a lignin-oxidizing enzyme involved in the recalcitrant C, was measured. The experimental procedure for soil enzymatic activity determination was described in Prieto-Rubio et al. (2023). Per each sample, soils were incubated with two specific buffers (Tris-maleate 40 mM, pH 8 for leucine aminopeptidase; Tris–acetate 10 mM, pH 11 for alkaline phosphatase; and Tris–acetate 10 mM, pH 4.5 for the rest of enzymes, 1 g of soil per buffer) at 100 rpm and 25ºC. After incubations, photometric assays were carried out for the laccase activity determination (at 415 nm), and fluorogenic ones for the rest of enzymatic activities (at 355/460 nm of excitation/emission) by using a Victor microplate reader (Perkin-Elmer Life Sciences, Massachusetts, USA). Enzymatic

activities were expressed at pmol x mg soil^{-1} x h^{-1} x SOM^{-1}and log-transformed to fit them into a Gaussian distribution.

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Supplementary Figures

Figure S1. Variations in ectomycorrhizal (ECM) fungal OTU richness and network metrics by season (autumn, spring), at each site. Analysis of variance done in R as: aov(log(community parameter) ~ Season). P-value (or ns when p > 0.10) is indicated over each comparison autumn-spring. OTU richness = number of OTUs composing the co-occurrence network. Fungal 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 = network complexity by discarding OTU richness intrinsic variation.

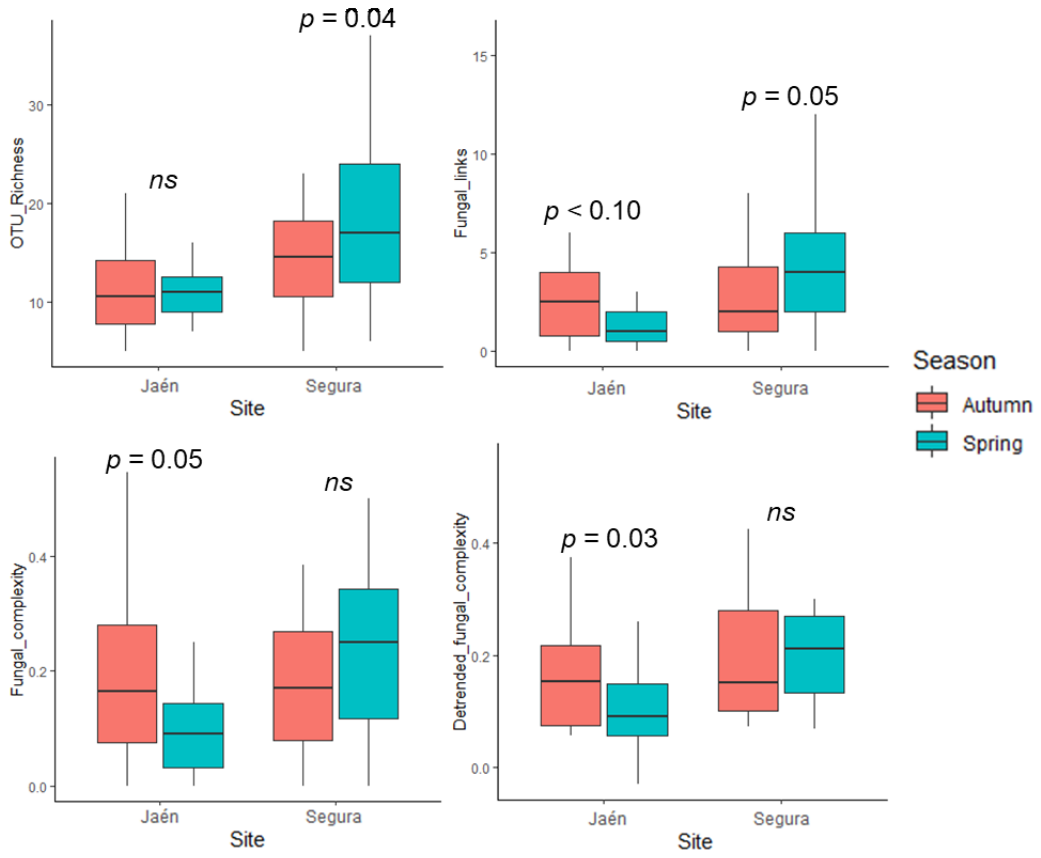

Figure S2. Variations in ectomycorrhizal (ECM) fungal OTU richness and network metrics by study site (Jaén, Segura). Analysis of variance done in R as: aov(log(community parameter) ~ Site). P-value is indicated over each comparison Jaén-Segura. OTU richness = number of OTUs composing the co-occurrence network. Fungal 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 = network complexity by discarding OTU richness intrinsic variation.

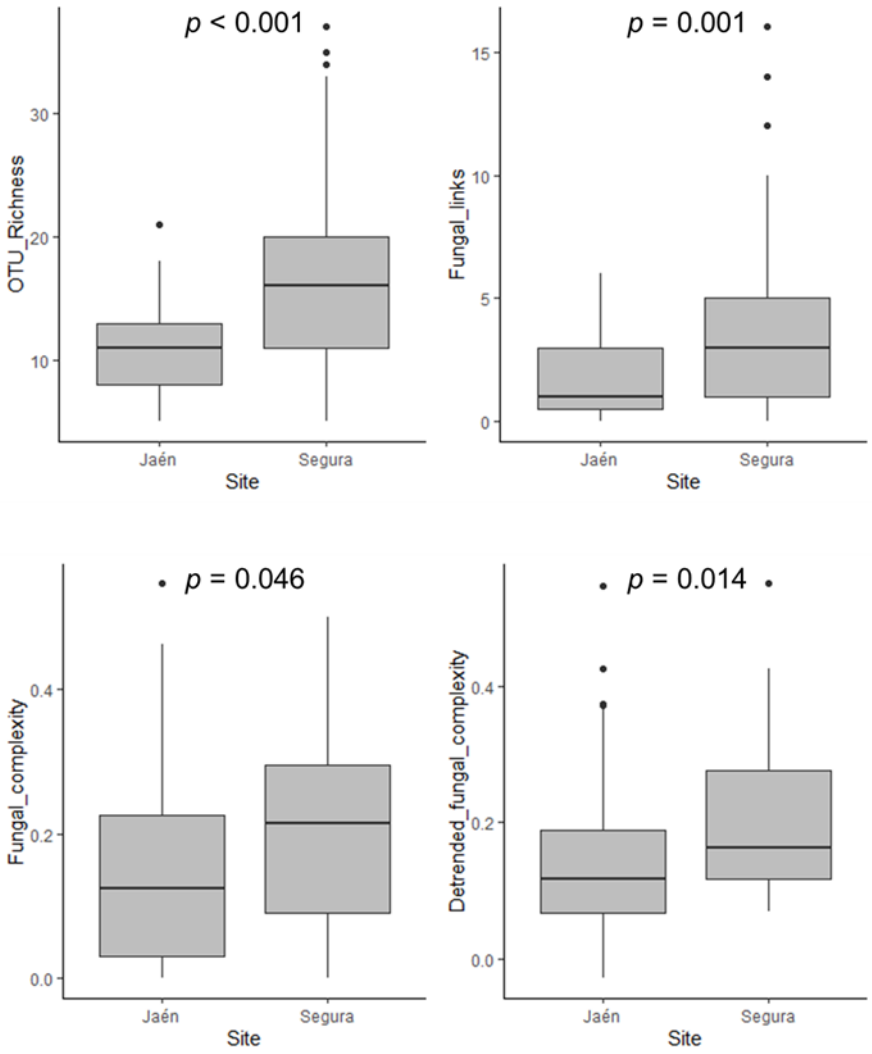

Figure S3. Correlations between co-occurrence network parameters and soil properties across the study sites (Jaén, Segura). A Spearman correlation test with Bonferroni adjustment was carried out per each site dataset. Positive (red) and negative (blue) correlations are shown, indicating those significant, i.e., $p < 0.05$, with '*' and marginal, $p < 0.10$, with no symbol. OTU richness = number of OTUs composing the co-occurrence network. Fungal 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 = network complexity by discarding OTU richness intrinsic variation. SOM = soil organic matter; GM = gravimetric soil moisture.