Indirect host effect on ectomycorrhizal fungi: Leaf fall and litter quality explain changes in fungal communities on the roots of co-occurring Mediterranean oaks

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1. Introduction

Mycorrhizal symbioses are essential for oak trees for acquiring nutrients under natural conditions (Smith and Read, 1997). Ectomycorrhizal (ECM) fungi supply plants with water and nutrients by increasing their foraging area and absorbing efficiency, and they provide an ample range of other beneficial effects as well, in exchange for photosynthesised products (Schultendiebel and Polle, 2002; Frey-Klett et al., 2005; Egerton-Warburton et al., 2007; Finlay, 2008). ECM communities contain a high diversity of fungal taxa (Taylor and Alexander, 2005), which are associated with a variety of functional strategies that contribute to forest ecosystem stability and functioning (Perry et al., 1989; Nara, 2006).

During the last decade, numerous studies have tried to unveil the role that natural factors, such as environmental conditions and host plant community composition, have on the assemblage of ECM communities (Conn and Dighton, 2000; Dickie and Reich, 2005; Buée et al., 2007). Soil abiotic conditions, namely, soil moisture, pH and nutrient availability, influence the performance and assemblage of fungal species (Brearley, 2006; Cavender-Bares et al., 2009). Host trees may directly affect the assemblage of their mycorrhizal community by exerting a selection for mycorrhizal species (Ishida et al., 2007; Tedersoo et al., 2008).

In a recent study Morris et al. (2008) studied the separate effects of soil conditions and host tree species on the composition of ECM communities in a California mixed-oak forest and concluded that both explained a significant proportion of the variation in ECM species distribution. These effects have usually been investigated independently despite the fact that host trees, acting as ecosystem engineers, may also indirectly shape ECM communities through their ability to modify the abiotic conditions of their environment (Jones et al., 1994; Bennett et al., 2009). The magnitude and direction of these changes are species-specific, and they can be mediated by litter quality and biomass, root exudation and nutrient uptake (Gobran et al., 1998; Mitchell et al., 2007). The complex interactions among host plants, environmental conditions and fungal communities are difficult to disentangle and the relative importance of the indirect host effects on this mutualistic relationship remains unclear.

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Oak forests of southern Spain are an important economic and social resource at the same time that they are a hotspot of endemic and relict plant species (Médail and Quézel, 1999; Anonymous, 2005). Despite the well-known dependence of oaks on mycorrhizal fungi, only a limited number of studies have been done on the belowground ECM communities in oak forests (Walker et al., 2005; Buée et al., 2007; Avis et al., 2008), and even fewer have been carried out in areas with seasonally dry Mediterranean-type climates (Richard et al., 2005; Smith et al., 2007; Morris et al., 2008). In this study we aimed to investigate the importance of the indirect effects of host tree species on the ECM community. For that purpose we studied the ECM fungal community on the roots of two coexisting oak trees, the sclerophyllous evergreen Quercus suber (cork oak) and the winter-deciduous Quercus canariensis (Algerian oak), under the Mediterranean-type climate of southern Spain, using PCR-based molecular methods.

The research objectives of this study were: i) to assess the diversity and structure of the ECM communities on the roots of these two co-occurring Quercus species in two Mediterranean mixed-oak forests, using DNA-based identification techniques; ii) to analyse the relationships between the distribution of ECM species and the measured litter and soil variables by using multivariate methods; iii) to evaluate the extent to which the composition and diversity of the ECM community may be explained by the soil conditions, the host identity and the litter-mediated changes in the topsoil environment (soil and litter) and iv) to compare among several alternative causal models explaining the plant–soil–fungal interactions, in order to test the hypothesis that the studied oak host species, through the differences in their leaf fall quality, may produce key changes in the litter and topsoil chemical composition that in turn, may affect the ECM community assembly.

2. Materials and methods

2.1. Study area and forest sites

The study area is located in the Aljibe Mountains, in the south of Spain. The region has a rough topography, with the highest peak reaching 1092 m a.s.l. The bedrock dominated by oligo-miocene sandstone originates acidic, nutrient-poor soils (Palexeralfs; Soil Survey Staff, 2006) frequently interspersed with layers of marl sediments that yield soils richer in clay (Haploxererts; Soil Survey Staff, 2006). The climate is of the sub-humid Mediterranean-type with most rainfall (95%) occurring from October to May. See detailed descriptions of the area in Ojeda et al. (2000), and Anonymous (2005).

The vegetation is dominated by evergreen cork oak (Q. suber L.), mixed with the winter-deciduous Algerian oak (Q. canariensis Willd.), which is locally abundant in the valley bottoms (Uribeta et al., 2008). The arborescent shrubs Phillyrea latifolia L. and Pistacia lentiscus L. dominated the understorey. The area has been protected since 1989 as “Los Alcornocales” (meaning “the cork oak forests”) Natural Park (Anonymous, 2005).

Two structurally different forest sites, 40 km apart, were selected within the study area. The site at San Carlos del Tiradero (36° 9’ 46” N; 5° 35’ 39” W), hereafter called “Forest”, was located in the south of the Park near the coast at 335–360 m a.s.l. on a NE facing slope. The mean annual rainfall is 964 mm, and the mean annual air temperature is 16.6°C, with a minimum of 4.1°C. The Forest stand had a high density of trees (769 stems ha−1) with a basal area of 47 m2 ha−1 (estimated on trees with dbh > 1.6 cm).

The other site, at La Sauceda (36°31’54”N; 5°34’29”W), hereafter called “Woodland”, was located inland, in the north of the Park, at 530–560 m a.s.l. on a NW facing slope. It has a mean annual temperature of 15.5°C, with a minimum of 1.8°C and a mean annual rainfall of 1470 mm. The woodland tree density was relatively low with 219 stems ha−1 and a basal area of 22 m2 ha−1 (Pérez-Ramos et al., 2008).

2.2. Sampling design

At each forest site (Forest, w: Woodland), six adult individuals of Q. suber (S) and six of Q. canariensis (C) located in a matrix of coexisting oak species and spread across approximately 1 ha were selected. Thus a total of 24 oaks were sampled that can be grouped into four categories (Cf, Cw, Sw, Cc; each with six replicates) of two combined factors: oak species and forest site. The selected trees were estimated to be more than 50 years old.

Leaf fall, litter, topsoil (~1400 cm3, 0–25 cm depth) and subsoil (~1400 cm3, 25–50 cm depth) were sampled beneath the canopy of each selected oak at approximately 2 m from the trunk in November 2006. Annual leaf fall was collected by four traps (50 cm diameter) located under each tree. The contents were removed, and the leaves were separated and dried. Two 30 × 30 cm quadrats were sampled to assess the litter biomass, by the harvesting and drying method (expressed as kg dry mass m−2). Both leaf fall and litter samples were composited from leaves of one oak species since the closest neighbours of the selected trees were individuals of the same species and they had no significant understorey cover under their canopy. Once the litter layer was removed, cores of soil were extracted with a cylindrical auger; four samples of topsoil (0–25 cm) and four of subsoil (25–50 cm) were taken under each oak tree in the four cardinal directions and pooled into single representative samples.

Superficial roots (~0–15 cm depth) approximately equal in length (~20 cm) were taken from each selected tree, close to the litter and soil sampling points, in November 2007. Root samples were kept moist in sealed plastic bags and transported inside an ice-box to the laboratory, where they were stored at 4°C. Within two days, each root system was examined under a binocular microscope, and 20–22 ectomycorrhizal root tips from each tree were randomly picked free of debris, removed with tweezers and individually stored frozen in 100 μl of 2× CTAB buffer.

2.3. Leaf fall, litter and soil analyses

Samples of leaf fall and litter were dried at 70°C, weighed, and ground for chemical analysis. Soil samples were dried (30°C for 48–72 h) and crushed to pass through a 2 mm sieve. Soil acidity (pH) was determined potentiometrically in a 1:2.5 soil:HzO solution. The percentage of soil carbon was estimated using a Total Organic Carbon Analyzer (TOC-Ves). The available P was estimated using the Bray 1 method (Bray and Kurtz, 1945). The total concentration of several macro-nutrients (Ca, K, Mg, P and S) in plant tissues and soils was determined by acid digestion with nitric acid or aqua regia followed by ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry). Plant and soil nitrogen was determined by using Kjeldahl digestion and subsequent determination of nitrogen with a micro-Kjeldahl digestion and subsequent distillation—titration in a Bran-Luebbe Autoanalyzer. See methods in Allen (1989).

2.4. Ectomycorrhizal DNA extraction, amplification and sequencing

From each ectomycorrhiza sampled, DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Charbonnieres, France). The CTAB extraction buffer was removed, and the ectomycorrhizas were rinsed with sterile water. Two hundred microliters of Nuclei Lysis Solution were added. Samples were ground into slurry using a micro-homogeniser with sterilised tips and then incubated for 15 min at 65°C. Subsequently, 67 μl of protein precipitation reagent was added to the samples. The protein was precipitated overnight at 4°C and the DNA supernatant was collected and purified with Wizard SV Gel and PCR Clean-up Kit (Promega, Charbonnieres, France). DNA was eluted in 50 μl of water and stored at −20°C. DNA purity and quality were checked by gel electrophoresis and the DNA concentration was determined by NanoDrop Spectrophotometer (ND-1000, SpectraMax, USA).

Sequences were demultiplexed with a Barcodes分离器 from Macrogen. The libraries were sequenced in both directions on an Illumina MiSeq platform at Macrogen. The data was processed using QIIME pipeline (Caporaso et al., 2010) using default parameters. The reads were filtered and clustered into operational taxonomic units (OTUs) based on a 3% distance using UCLUST (Edgar, 2010). Chimaeras were removed using實施ChimaeraFilter. Each OTU was annotated with a representative sequence using SG-Classifier and the Ribosomal Database Project (RDP). The OTUs were classified at the genus level.

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solution was added and vortexed. The mixture was centrifuged at 14,000 rpm for 10 min, and the supernatant containing DNA was moved to a fresh tube. The DNA was precipitated by isopropanol and resuspended overnight at 4 °C in nuclease free water.

Following extraction, the internal transcribed spacer regions I and II and the nuclear 5.8S rRNA gene were amplified using the primer sets ITS-1F/ITS-4B (Gardes and Bruns, 1993) or ITS-1F/ITS-4 (White et al., 1990) depending on amplification success. The PCR mixture for one sample was composed of 15.8 μl of nuclease free water, 5 μl of 10× Buffer, 0.25 μl of each primer (5 μM), 5 μl of deoxynucleotide triphosphate (5 mM) and 0.2 μl of GoTaq. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 1 min; 35 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min; and a final elongation at 72 °C for 10 min. PCR products were assessed by electrophoresis (30 min at 100 W on 1% agarose gels) before sequencing. Gels were stained with ethidium bromide and photographed under ultraviolet light. Unsuccessfully amplified samples were subjected to multiple attempts of PCR at various concentrations of template. The sequencing of the final amplification products was done by MilleGen (Labège, France). Nucleotide sequences were manually edited with the freeware program Chromas Lite 2.01 (http://www.technelysium.com.au/chromas.html).

2.5. Ectomycorrhizal identification

Ectomycorrhizal “operational taxonomic units” (OTUs) (Blaxter et al., 2005) were determined as follows: First, edited sequences were examined by BLAST searches against GenBank (http://www.ncbi.nlm.nih.gov/) and the UNITE database (http://unite.zbi.ee/; Köljalg et al., 2005).

Few sequences shared 97% or greater similarity with any of the species deposited in GenBank or UNITE. This cut off level was established based on studies using the ITS region for ECM species identification that have shown that error rates generated by PCR, sequencing and interspecific variability are generally < 3% (Nilsson et al., 2006). Due to the limited number of species identified by BLAST search the samples were grouped by the closest matching genus. For each genus group, sequences were aligned and analysed with MEGAS4 (http://www.megasoftware.net/), and the p-distance (% of different nucleotides) was calculated. This measure, although simple, is adequate when studying closely related distances (Nei and Kumar, 2000). Sequences with < 3% of a difference in p-distance were ascribed to a single OTU. The complete name for a species was only given to those OTUs that match a GenBank species sequence by ≥ 97%.

In order to test the grouping and identification of OTUs, phylogenetic trees were generated that included all of the sequences from the same genus and some identified sequences from GenBank as external taxonomic benchmarks. Two types of methods were used for the phylogenetic analysis: 1) the neighbourhood joining method with Kimura-2 distances and confidence assessed by bootstrapping with 500 replicates, and 2) the maximum parsimony method with a heuristic search, tree bisection-reconnection bootstrapping with 500 replicates, and 2) the maximum parsimony method with a heuristic search, tree bisection-reconnection bootstrapping with 500 replicates, and a non-parametric alternative for t-test. Occurrence was counted as the number of trees in which fungal OTUs were found.

Species (more precisely OTU) richness (S) was measured as the number of species in a defined sampling unit. Rarefaction analysis was used to estimate the number of species expected in each sample if all samples were of a standard size, thus overcoming the effect of unequal numbers of identified mycorrhizas. The dominance of the community was assessed using Simpson’s diversity index (1-D). Fisher’s alpha (α) was also used to measure diversity because of its good discriminant ability and the fact that it is hardly influenced by sample size (Magurran, 1988). All indexes were calculated using Biodiversity Pro V.2 (http://www.sams.ac.uk/research/software).

The taxonomic distinctness index (Δ*), as defined by Warwick and Clarke (1995), was used to estimate the phylogenetic relatedness of the ECM species. It was calculated, based on the topology of a taxonomic tree, as the average path length between any two randomly chosen individuals, conditional on them being from different species. The phylogenetic structure of the ECM communities was statistically assessed using UNIFRAC (Lozupone et al., 2006). A phylogenetic tree was constructed using the neighbour-joining method and both the UniFrac distance metric (Lozupone and Knight, 2005), which is calculated based on differences in the tree branch length, and the P test (Martin, 2002), which estimates similarity between communities based on tree topology, were used to detect differences in the ECM community composition between host species and forest sites.

The similarity of ectomycorrhizal fungal communities between oak trees and between sites was assessed with the Sorensen index. In order to take the distribution of species into account, similarity was also estimated based on abundance with the percentage similarity coefficient according to Jongman et al. (1995).

Two-way factorial ANOVAs using site and host species as categorical factors were performed for each of the measured environmental variables to ascertain if the overall environmental conditions differed significantly under the studied oak species and in both forest sites. When necessary, transformations (logarithmic, square root) were performed to satisfy the necessary assumptions (normality, homoscedasticity). The relationship between the ECM community composition and environmental conditions was analysed by a direct gradient analysis method, the Canonical Correspondence Analysis (CCA), using the Canoco for Windows 4.5 package. This unimodal ordination method was preferred over a linear one (redundancy analysis) since the unconstrained ordination (detrended correspondence analysis, DCA) of the species distribution data showed that they were largely heterogeneous, defining a long main gradient (ter Braak and Smilauer, 2002). Some rare species (singleton) distorted the analysis; producing deviant samples (outliers); thus, we performed the CCA analysis without singletons. Nevertheless, we compared the results with those derived from the analysis done with the complete set of ECM species. Species data were log (x + 1) transformed to reduce skewness.

In order to investigate the overall patterns of covariation between the measured variables a d-sep method of path analysis

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(Shipley, 2000) was used to test for alternative causal models
linking the tree (leaf, leaf fall, litter, host identity), soil (topsoil and
subsoil) and the ECM fungal community. Each alternative model
teinates a series of causal assumptions related to the implications of
the model for the variances and covariances of the variables that
can be tested against the obtained empirical data. A model fits the
data when these assumptions can not be falsified (i.e. $p > 0.05$). As
a surrogate of the overall ECM community composition we used the
main independent gradient of variation extracted with an uncon-
strained correspondence analysis (CA) on the ECM species data. The
CA analysis guaranteed no influence of the environmental variables
on gradient selection. Because most variables were not normally
distributed we used Spearman correlations in the analysis, for
which data was converted to rank.

Since different sets of repeated tests were performed throughout
the study a type I error inflation was expected (see García, 2004). To
cope with this increased frequency of spurious results, while min-
imising power losses associated to Bonferroni-related procedures, we
controlled the expected proportion of ‘false positives’ using an FDR (False Discovery Rate) controlling procedure as recommended by García (2003).

3. Results

3.1. ECM community in a Mediterranean forest

A total of 69 different species (OTUs) were identified from the
root tips sampled, belonging to 24 genera and 18 families. A
detailed list of the identified OTUs is presented in the Supporting
Information Table S1. Basidiomycetes dominated the fungal
communities on roots of both oak trees (>90% of all identified
mycorrhizas) and were present in virtually all sampled trees (23 out
of 24). Ascomycetes were scarce, accounting for only 10 mycor-
rhizas (5% of total abundance), but they were relatively widespread,
occuring in 10 of the 24 trees sampled and in both oak species and
forest stands. Both fungal groups were similarly distributed among the
two oak species and sites ($p > 0.85$; based on Yates corrected $\chi^2$ test). Thelephoraceae was the most diverse family (comprising 28% of the species), followed by Russulaceae (22%) and Cortinariaceae (17%). Eighteen of the 24 identified genera were comprised of only two species. *Tomentella* and *Russula* were the most widespread and abundant taxa. The most frequent OTUs were *Lactarius chrysorhoeus* (found on seven trees) and *Cenococcum geophilum* (on six trees), which attained 6% and 3% of the total abundance, respectively. Two-thirds of the identified ECM were singletons, occurring only in one sample.

ECM fungal species with epigous fruiting bodies were the most
diverse (55% of the species) and abundant (60% of identified
mycorrhizas) followed by resupinate fungi that attained 27 species
and 33% of the total abundance. Hypogeous fungi were scant (3%),
and they were rarely found (in 5 oak trees). The frequencies of fruiting habits varied between sites and oak species (Yates cor-
rected $\chi^2$, $p < 0.03$).

The total number of ECM species associated with oak trees was
similar for the two *Quercus* species in both study sites (Table 1).
Rafarefaction analysis indicated a lower diversity of ECM species in
the Forest than in the Woodland site, irrespective of the host
species (Fig. 1). Other indices, such as the Simpson’s diversity index
or Fisher’s alpha, confirmed this tendency (Table 1). Equal numbers of families (14) and genera (18) were found on roots of both oak
species; however, the taxonomic distinctness values were lower for
the fungal species colonising the roots of the Algerian oak (*Q. canariensis*) in the Woodland. These results were in agreement
with the UniFrac analysis, which suggested that there were significant community shifts between environments overall (Uni-
Frac metric $p < 0.0026$). Lineage specific analysis revealed that these differences were primarily because of the tomentelloids and rus-
suloids which differed significantly ($p < 0.0000$) between the four
oak-site environments. The ECM community on the roots of *Q. canariensis* in the Woodland site (Sw) was dominated by
*Tomentella* (53% of identified mycorrhizas), followed by *Cortinarius*
(15%). In contrast, *Russula* was the most abundant taxon ($\approx$ 35% of the symbionts) on the other three species-site combinations (Cf, Sw
and Sf). The community on *Q. suber* in the Forest site (Sf) was
dominated by three russuloids, *Russula* (30%, already mentioned),
*Lactarius* (14%) and *Macowanites* (10%) (Fig. 2).

Among the 69 identified fungal species, only 13 were found on
both *Quercus* species, e.g.* C. geophilum*, *Otidea* sp1. and *Byssoc-
corticium atrorivens*, while 29 were recorded exclusively on
*Q. canariensis* roots, and 27 species occurred only on *Q. suber*.
The limited number of common fungal species between trees resulted
in low Sorensen similarity values that ranged from 0.13 to 0.39
(Table 1). Communities from Sw and Cf roots were 35% of similar,
and both had about a 17% similarity with Sw. The most dissimilar
ECM community was that on the roots of *Cw* ($\approx 6\%$), which only
shared 4 species with the other groups.

3.2. Environmental heterogeneity: oak species and site factors

There were significant differences between forest sites and oak
host species for some of the measured environmental variables
(Table 2). Soil Ca and pH exhibited highly significant ($p < 0.005$)
additive effects for both site and oak species. It is remarkable that

| Table 1 | Diversity measures of the ECM fungal communities for the combinations of oak host species (*Q. suber* and *Q. canariensis*) and forest sites. Cw: *Q. canariensis*-Woodland, Cf: *Q. canariensis*-Forest, Sw: *Q. suber*-Woodland, and Sf: *Q. suber*-Forest. |
|---|---|---|---|---|
| Diversity measure | Woodland | Forest | All trees |
| | *Q. canariensis* | *Q. suber* | *Q. canariensis* | *Q. suber* | *Q. canariensis* | *Q. suber* |
| Species richness (S) | 22 | 25 | 23 | 23 | 69 |
| Species density (average spp./per tree) | 4.0 | 4.8 | 4.5 | 6.3 | 4.9 |
| Simpson diversity index (1-D) | 0.954 | 0.954 | 0.873 | 0.935 | 0.965 |
| Rarefaction species richness (n = 38) | 22.00 | 21.53 | 17.35 | 17.45 | 35.4 |
| Fisher’s alpha (\( \alpha \)) | 21.8 | 21.0 | 14.3 | 11.9 | 35.4 |
| Taxonomic distinctness (\( \Delta \)) | 2.97 | 3.45 | 3.28 | 3.25 | 3.44 |
| Singleton species | 20 | 19 | 21 | 15 | 46 |

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litter Ca was the only factor showing both high significant additive and multiplicative (site × oak) effects.

Litter and topsoil sampled under the canopy of the winter-deciduous species (Q. canariensis) were significantly richer in Ca, and the soils were less acidic than those sampled under the evergreen oaks (Table 2). These results were consistent with the differences found in the amount and composition of the leaf fall collected during a whole year under both species (Table 2, third panel), in which Ca was the only element significantly enriched (51% higher) in the leaf fall from the winter-deciduous species. The level of this leaf fall enrichment essentially depended on the oak species because the effects of site and site × oak were not significant.

Regarding the relationships among environmental variables, some very high ($r \geq 0.9$) correlations between litter Ca, soil pH and soil Ca contents were detected, as well as high correlations ($r = 0.8$–0.9) between other soil and litter variables (Table 3).

3.3. ECM community–environment relationships

The distribution of the ECM species was explained by significant conditional effects of the forest site ($p < 0.023$), the host oak species ($p < 0.038$) and the site × host interaction ($p < 0.036$), as shown by the factorial CCA. The overall CCA factorial model explained 19.6% of the total ECM species variance and was highly significant ($p < 0.0023$).

When the measured topsoil variables were used as predictors of the ECM species distribution alone, only two variables showed highly significant ($p < 0.01$) marginal effects: soil pH ($p < 0.0007$) and soil Ca content ($p < 0.0041$). Because of the very high correlation between these two variables (Table 3) only the pH had a significant conditional effect and thus was retained as the best predictor. This soil variable alone was able to explain 8.2% of the overall ECM species variance.

On the other hand, when the litter variables were used in the CCA analysis only litter Ca showed a highly significant ($p < 0.01$) conditional effect as a predictor of the ECM community structure. Furthermore, when both the soil and litter variables were included in the analysis, the model based on litter Ca content alone ($p < 0.0002, 8.4%$ overall variance explained) outperformed all other CCA models based on any combination of the remaining measured soil and litter variables, including soil pH. The close relationship between litter Ca and soil pH (Table 3) and the better predictive performance of the litter Ca explained why only this variable was retained as a meaningful predictor of the ECM community structure.

In order to ascertain whether the hypothesised interactions between host species, soil and litter conditions and ECM fungal communities had a significant empirical support we used d-sep analysis to test different alternative causal models which underlying hypothesis were: 1) Main variations in the ECM communities may be explained by soil changes derived only from differences in intrinsic soil properties (subsoil), 2) Only host identity affects the ECM community composition by means of a direct genetic specificity, 3) There is a species indirect effect via differential leaf, leaf fall and litter quality that explains the main ECM variation patterns and 4) The combination of direct and indirect species effects and the primary soil properties drives the ECM community composition. All path diagrams corresponding to the proposed models are shown in Fig. 3. Calcium concentration in leaves, leaf fall, litter, topsoil and subsoil were used as surrogate variables in the models.

Table 4 includes the results of the analysis of the tested causal models. Only models explicitly considering the indirect effects of host species on ECM communities, via changes in litter (or litter and topsoil) composition matched the overall available empirical evidence (Fig. 3). Nevertheless the model based on simultaneous direct and indirect host influence was clearly more consistent with the empirical evidence ($p = 0.21$) than the one supported only by indirect effects ($p = 0.06$). All the models considering the independent influence of primary soil properties on the ECM communities were rejected.

3.4. Environmental conditions and diversity of the ECM communities

The overall mycorrhizal species richness increased significantly with soil acidity ($r = -0.58, p < 0.003$). Negative relationships were found between the number of ECM genera and families and the litter Ca concentration ($r = -0.43, p < 0.034$) indicating a significantly lower taxonomic diversity with increasing Ca availability and soil pH.

The distribution of taxa according to their fruiting habit was also significantly related to environmental conditions. The diversity and
abundance of epigeous populations was negatively correlated with Ca in litter \( (r = -0.50, p < 0.03) \) and soil pH \( (r = -0.57, p < 0.03) \). The opposite pattern was observed for resupinate species, for which richness rose along the gradient of Ca in litter. The mean, standard error and \( r \)-values that remained significant after applying a tablewise FDR correction are in bold, and those that are additionally significant on a per-test basis \( (p < 0.05) \) are in italics.

### Table 2

The mean, standard error and \( r \)-values resulting from two-way ANOVA analyses for environmental and complementary variables measured in the topsoil, litter (L-) and leaf fall (F-) samples collected under the canopy of the two oak host species in the two forest sites. Complementary information on green leaves and subsoil is provided in the lowest panel. The \( r \)-values that remained significant after applying a tablewise FDR correction are in bold, and those that are additionally significant on a per-test basis \( (p < 0.05) \) are in italics.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unit</th>
<th>Quercus canariensis</th>
<th>Quercus suber</th>
<th>Site</th>
<th>Oak</th>
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<td>Forest</td>
<td>Woodland</td>
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<td>0.03</td>
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<td>0.00</td>
<td>0.12</td>
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<td>11.0</td>
<td>1.2</td>
<td>3.2</td>
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### 4. Discussion

#### 4.1. ECM fungal communities in a Mediterranean forest

We have found that southern Spain oak forests harbour a relatively high ECM fungal species richness: 69 OTUs belonging to 24 genera and 18 families were identified in the roots of two oak species in two forest sites. These richness values are in accordance with others found in oak forests (ranges from 39 to 79; [Avis et al., 2003; Walker et al., 2005]), although lower than the maximum values of 95–140 taxa recorded in some Mediterranean forests ([Richard et al., 2004; Morris et al., 2008]). The species-area curve obtained here (Fig. 1) indicates that the local ECM fungal biodiversity must be even higher. We found a large number of rare taxa (46 singleton species) that is a common feature in ECM fungal communities ([Köljalg et al., 2000; Avis et al., 2008]).

Thelephoraceae (28% of the species), Russulaceae (22%) and Cortinariaceae (17%) were the most diverse and abundant families in the ECM fungal communities, as has also been found in other forests ([Gardes and Brun, 1996; Richard et al., 2005; Riviere et al., 2007]). In a similar study, [Morris et al. (2008)] investigated the ECM fungal community on the root of two coexisting oak species in a California woodland, and found a large abundance of Ascomycota (40%) and hypogeous fruiting fungi (~25%). In contrast, a low abundance of this group was recorded in Mediterranean Quercus forests of south Spain (this study) and Corsica ([Richard et al. (2005)]). These strong differences between ECM fungal communities on similar oak species and under Mediterranean-type climate, but in distant biogeographical regions (California versus Mediterranean Basin), are worth to be studied in more detail. The Ascomycota species *C. geophilum*, which is a known associate of *Quercus* roots ([Dickie et al., 2004; Walker et al., 2005]), only represented 3% of the total abundance in the forests studied. The relative humidity and high precipitation level in the study sites may negatively impact the colonisation and spread of this drought tolerant fungus ([Pigott, 1982]). In addition, the small size of *C. geophilum* ECM tips may cause the unsuccessful amplification of their DNA and thus, result in their under-representation ([Smith et al., 2007]).
4.2. Indirect effects of host species on ECM fungal communities

One of the main findings of this study is that the oak host species may indirectly affect ECM fungal communities through their leaf fall and litter quality, as is suggested by the tested causal models. In general, the species-specific effects of trees on biogeochemical cycles and ecosystem processes have long been recognised (Zinke, 1962; Hobbie, 1992; Finzi et al., 1998). In the two forests studied the calcium content in litter and soil, together with soil pH, emerged as the most influential variables for mediating indirect host species effects on ECM fungal communities since: i) they were the only significant variables explaining the ECM fungal community patterns even when other potentially influential factors (soil C, N, and P) were also studied; ii) they exhibited a highly significant oak species effect irrespective of the homogeneous subsoil conditions; iii) significant differences in the leaves and leaf fall Ca that were exclusively related to the oak species were observed before the leaf fall interacts with the soil surface; and iv) they are conceptually related, since increased Ca inputs can raise exchangeable base saturation and mitigate soil acidity. Accordingly, the winter-deciduous oak (Q. canariensis) was able to pump from the soil significantly more Ca than the coexisting evergreen species (Q. suber) leading to a top-down cascading effect by which the relative Ca enrichment in the materials collected under the winter-deciduous species in comparison to the evergreen Q. suber increased from leaf fall (51% higher) to litter (81% higher) to topsoil (251% higher). We suggest that the differences in leaf fall Ca-levels, and their yearly accumulation and incorporation into the litter, yield increasingly distinct litter and topsoil, thus producing selective environmental conditions that shape the ECM fungal community. The observed shift in the dominant taxa from Russula to Tomentieloids was the most outstanding change associated with the oak-induced variation in the environmental conditions and it was observed in the two forest sites despite their contrasting environmental conditions.

Leaf fall calcium content is a tree species-specific trait that depends on its uptake, allocation and retention capacity; thus, even species growing under similar subsoil nutrient supplies, as it occurred in the studied forests, in time, distinctly modify their soil abiotic environments (Reich, 2005). Leaf fall Ca determines litter Ca, which has a strong relationship with forest floor biogeochemical dynamics such as soil acidity, base saturation, C and N contents, decomposition rates and soil heterotrophic community composition (Reich, 2005; Hobbie et al., 2006). Since soil pH was closely correlated to calcium levels in soil and litter, and since it was also a significant predictor for ECM fungal species distribution, calcium-induced changes in soil acidity seem to play a main role in determining the observed differences in the fungal communities between environments. The relationship between shifts in ECM fungal species communities and soil pH has been previously described: Soil acidity seems to have a species-specific effect on the production of fruiting bodies (Agerer et al., 1998). In vitro experiments have shown that substrate pH affects the growth capacity (Hung and Trappe, 1983), mycelial density (Rosling et al., 2004), colonisation potential (Erland and Soderstrom, 1990) and enzymatic capabilities (Courty et al., 2005) of mycorrhizal species. Changes in the performance of species under different pH levels affect their competitive abilities (McAfee and Fortin, 1987), altering their relative abundance in the community.

Table 4
Shipley's d-sep test results of different causal models which could potentially explain the observed covariance patterns among host species (Q. canariensis, Q. suber), Ca content in different forest compartments (green leaves, leaf fall, litter fall, subsoil and topsoil) and ECM fungal community structure (summarised by the main axis extracted by correspondence analysis from the ECM data).

<table>
<thead>
<tr>
<th>Model</th>
<th>Chi-sq</th>
<th>d.f.</th>
<th>p</th>
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<td>1</td>
<td>92.05</td>
<td>30</td>
<td>0.0000</td>
<td>Direct effect of soil intrinsic properties</td>
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<tr>
<td>2</td>
<td>32.80</td>
<td>20</td>
<td>0.0354</td>
<td>Direct effect of host genetic speci</td>
</tr>
<tr>
<td>3</td>
<td>28.30</td>
<td>18</td>
<td>0.0577</td>
<td>Indirect effect of host species via leaf fall and litter quality</td>
</tr>
<tr>
<td>4</td>
<td>20.16</td>
<td>16</td>
<td>0.2131</td>
<td>Direct and indirect effects of host species</td>
</tr>
<tr>
<td>5</td>
<td>56.68</td>
<td>28</td>
<td>0.0011</td>
<td>Direct effect of soil intrinsic properties and indirect effect of host species</td>
</tr>
<tr>
<td>6</td>
<td>43.50</td>
<td>26</td>
<td>0.0171</td>
<td>Direct effect of soil intrinsic properties and direct and indirect effects of host species</td>
</tr>
</tbody>
</table>
were treated as an abiotic host-independent influence and were not related to host indirect effects through leaf fall or litter quality.

4.3. ECM fungal diversity and soil conditions

No significant changes in ECM fungal species richness were observed between oak species, and forest sites. However, the taxonomic diversity (number of genera and families) and the phylogenetic structure of the community significantly shifted.

Other studies have also found changes in species dominance from epigeous to resupinate (Peter et al., 2001) and from Basidiomycetes to Ascomycetes (Gehring et al., 1998) related to variations in soil conditions (e.g. increase in nutrient availability). Differences in the abundance and diversity of epigeous species between the roots of evergreen and deciduous coexisting oaks were also recently observed by Morris et al. (2008).

The lower ECM fungal taxonomic diversity recorded on the roots of Q. canariensis in the woodland site was mainly due to both the higher abundance of resupinate tomentelloid species and the disappearance of epigeous taxa. It is possible that the prevailing environmental conditions, i.e. higher litter, and soil nutrient content and pH, may be limiting for certain fungal species (e.g. Russula) and thus represent an environmental filter yielding a phylogenetic clustering of closely related “tolerant” species such as the tomentelloids.

Environmental selection for biological traits conferring toleration and selection for competitive interactions that promote functional diversification are two of the main processes that govern the phylogenetic structures of animal, plant and bacterial communities (Fox and Brown, 1993; Weiler and Keddy, 1995; Horner-Dovine and Bohannan, 2006). The relationships that we have found between ECM fungal taxonomic diversity and soil conditions in these oaks forests suggest that these complementary ecological processes might also drive the community structure of mycorrhizal fungi.

5. Conclusions

Our work suggests that leaf fall-mediated indirect host effects may play a critical role in determining the ECM fungal community assembly and taxonomic diversity. These effects could have been interpreted either as pure environmental effects (host-independent), if the relationships between soil properties and the host source materials (leaf fall, litter) had not been simultaneously investigated, or as a generic mixed host-site effect. If only a factorial multivariate analysis had been performed. To our knowledge this is the first attempt to investigate the indirect effect of host species on ECM fungal assemblies. Since this interaction had been previously unnoticed and seems to be superimposed to direct host effects, further work is needed to unveil its relative importance as a driving factor of the ECM fungal communities.

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Appendix. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.soilbio.2010.01.014

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