Research

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Maternal effects shape the seed mycobiome in *Quercus petraea*

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Summary

- The tree seed mycobiome has received little attention despite its potential role in forest regeneration and health. The aim of the present study was to analyze the processes shaping the composition of seed fungal communities in natural forests as seeds transition from the mother plant to the ground for establishment.
- We used metabarcoding approaches and confocal microscopy to analyze the fungal communities of seeds collected in the canopy and on the ground in four natural populations of sessile oak (*Quercus petraea*). Ecological processes shaping the seed mycobiome were inferred using joint species distribution models.
- Fungi were present in seed internal tissues, including the embryo. The seed mycobiome differed among oak populations and trees within the same population. Its composition was largely influenced by the mother, with weak significant environmental influences. The models also revealed several probable interactions among fungal pathogens and mycoparasites.
- Our results demonstrate that maternal effects, environmental filtering and biotic interactions all shape the seed mycobiome of sessile oak. They provide a starting point for future research aimed at understanding how maternal genes and environments interact to control the vertical transmission of fungal species that could then influence seed dispersal and germination, and seedling recruitment.

Introduction

In forests, seeds and seedlings are the stages that usually incur the highest mortality rates (Petit & Hampe, 2006). Seed predators, seedling herbivores, fungal pathogens and harsh environments (Moles & Westoby, 2004; Mangan *et al.*, 2010; Bagchi *et al.*, 2014; Bever *et al.*, 2015) are all known sources of mortality, with pathogens often a strong determinant of seed survival and seedling establishment. For example, the fungal pathogen *Ciboria batschiana*, the causal agent of acorn black rot, can damage up to 80% of oak seeds when conditions are wet (Prochazkova *et al.*, 2005). Seeds are also associated with many microbes with positive effects, including endophytic and epiphytic microorganisms that foster the growth of seedlings and protect them against natural enemies (Nelson, 2004; Links *et al.*, 2014; Leroy *et al.*, 2019). Understanding how pathogens and other microorganisms are acquired by trees, transmitted to their seeds, and influence seed germination and seedling development is crucial for improving our ability to predict and manage the regeneration of forest ecosystems.

Up to now, seed transmission of microorganisms has received far more attention in crops than in natural and managed forest ecosystems. In crops, microorganisms can be transmitted by the mother plant to its seeds during the floral and early seed development stages, through vascular tissues or contact between vegetative and reproductive organs (Maude, 1996). They can also be transmitted via the pollen of the father plant, insect vectors or bioaerosols (Frank *et al.*, 2017; Escobar Rodríguez *et al.*, 2018; Prado *et al.*, 2020). Once seeds fall on the ground, epiphytic microbial communities coalesce with microbial communities of litter and upper soil (Rillig *et al.*, 2015; Castledine *et al.*, 2020). Germinating seeds release molecules that attract soil microbes, surrounding themselves with a microbiologically active soil area called the spermosphere (Nelson, 2004; Schiltz *et al.*, 2015). The emergence of the plant radicle creates cracks in the seed tegument, enabling microbes to colonize internal tissues (Nelson *et al.*, 2018). These events lead to intense biotic interactions among microorganisms (Nelson, 2004) and drastic changes in seed microbiota composition and function (Ofek *et al.*, 2011; Yang *et al.*, 2017; Torres-Cortés *et al.*, 2018). Recent studies suggest that seed colonization by soil microorganisms represents the microbial acquisition that is most influential for seedling growth and health (Nelson *et al.*, 2018).

The microorganisms that are directly transferred from the vascular system of the mother plant to the offspring through seeds are termed vertically transmitted (Truyens *et al.*, 2015), while those recruited from external sources are termed horizontally acquired. Vertical transmission of microorganisms from mother to offspring is particularly important for plant population dynamics and evolution because it can provide progenies with microbial genes and functions that promote adaptation to local biotic and abiotic conditions (Schardl *et al.*, 2004). The microbial communities associated with the mother plant aboveground tissues can directly affect the phenotype and fitness of seedlings, by being transmitted to seedlings through seeds (Truyens *et al.*, 2015), or later through the litter (Christian *et al.*, 2017). They might also affect offspring indirectly by influencing seed provisioning and epigenome (Vivas *et al.*, 2015). Such effects of the maternal microbiota on the phenotype and fitness of the offspring are part of maternal effects. Maternal effects are often defined as the 'influences of the mother plant on the phenotype of her offspring via mechanisms other than the genetic information carried on chromosomes' (Weiner *et al.*, 1997; see also Roach & Wulff, 1987; Räsänen & Kruuk, 2007). Other authors have broadened this definition by including genetic effects into maternal effects. For instance, Wolf & Wade (2009) defined maternal effects as 'the causal influence of the maternal genotype or phenotype on the offspring phenotype'. We use this broad definition in the present article.

Maternally transmitted microorganisms can influence the dynamics of seed and seedling microbial communities. The latter are shaped, like all ecological communities, by four fundamental processes: selection by the abiotic environment (environmental filtering) and biotic interactions (biotic filtering), dispersal, ecological drift and evolutionary diversification (Vellend, 2010; Nemergut *et al.*, 2013; Ovaskainen *et al.*, 2017; Zhou & Ning, 2017). Some processes, such as the response to selection, are deterministic and depend on microbial functional traits (Torres-Cortés *et al.*, 2018). Other processes are partly or purely stochastic (Zhou & Ning, 2017; Rezki *et al.*, 2018) and generate divergences among communities occupying identical environments (Chase & Myers, 2011). Microbial community dispersal to the seed is a stepwise process because seeds are mobile so microorganisms can be recruited from a sequence of species pools. They can first be recruited from the mother plant's aboveground organs (but this is not the case in all plant species, as demonstrated by Sarmiento *et al.* (2017)). Next seeds can be colonized from bioaerosols (both while on the mother plant and once on the ground), as well as litter and soil pools. The microbiota initially transmitted by the mother plant, when it is present, is expected to have a large influence on seed community dynamics. Indeed, subtle differences in its composition can result in large differences in community composition among seeds, because initial differences can be amplified over time and space via microbial population growth and interactions (a phenomenon known as priority effects; Fukami, 2015).

The aim of the present study was to analyze the processes shaping the composition of seed fungal communities in natural forests as seeds transition from the mother plant to the ground for establishment. Little is known about fungal communities of tree seeds, and in particular about the vertical transmission of fungi from mother trees to their offspring; this transmission could be of importance to the adaptation of forest ecosystems to environmental changes and should be considered in forest tree planting (Vivas *et al.*, 2020). In previous work, both fungal pathogens and common endophytes were detected in the seeds of some tree species (Ganley & Newcombe, 2006; Qi *et al.*, 2012; Sarmiento *et al.*, 2017; de la Bastide *et al.*, 2019); however, the relative contribution of vertically and horizontally transmitted species to the assembly of the whole fungal community of seeds is not yet known in forest ecosystems. The extent to which fungal species transmitted by the mother tree persist after seed fall is not known either, although this is a prerequisite for their effect on offspring fitness. We therefore tested the following hypotheses in a major tree species of European temperate forests, sessile oak (*Quercus petraea*): (**H1**) seed fungal communities are shaped by maternal effects and environmental filtering, (**H2**) the similarity of seed fungal communities to the canopy environment decreases after seed fall because of horizontal acquisition of fungi from the ground, (**H3**) the lifestyle of fungal species influences their response to variations in the seed environment and (**H4**) biotic interactions at the seed scale are involved in seed protection against fungal pathogens. To test these hypotheses, we analyzed the seed mycobiome in four unmanaged oak populations located in the Pyrenees mountains (France). We collected oak seeds (i.e. acorns) in the canopy and on the ground beneath individual trees and used hierarchical models of species communities (HMSCs; Ovaskainen *et al.*, 2017) to quantify the ecological processes shaping seed fungal communities at each spatial level (oak population, mother tree and seed) and to generate hypotheses regarding microbial biotic interactions.

Materials and Methods

Sampling design

Samples were collected on 21 and 22 October 2015 in four intensively studied populations of sessile oak (*Q. petraea*; Vitasse *et al.*, 2011, Dantec *et al.*, 2015) growing naturally on mountain slopes in the Pyrenees, in the southwest of France (Fig. 1; Table 1). Two populations (Ade and Bager) are at an elevation of *c.* 400 m above sea level (asl) and the other two (Gèdre-Bas and Gabas) at *c.* 1200 m asl. The populations are *c.* 40 km apart (Fig. 1) and differ genetically (Alberto *et al.*, 2010). They consist of mature oaks *c.* 100 yr old with a trunk diameter at breast height of *c.* 45 cm, forming monospecific stands (Table 1). These populations were previously part of a long-term phenological monitoring (Vitasse *et al.*, 2009). The sampling date was chosen as close as possible to the fruiting peak at both elevations (reproduction is monitored weekly in those four populations; see Caignard *et al.*, 2017).

In each population, we selected three adult trees that had branches accessible for collection with a slingshot. For each tree, we collected four types of samples: four acorns from the canopy, taken from a branch brought down with the slingshot and

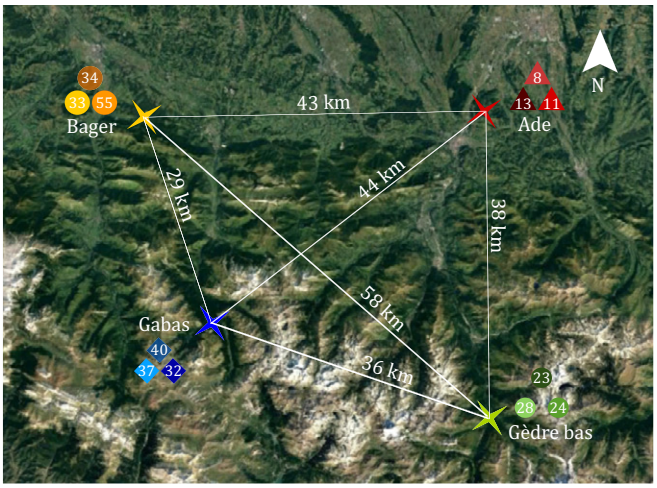

Fig. 1 Map of the oak populations sampled in the Pyrenees mountains (France). Four populations (Bager, Ade, Gabas, Gèdre-Bas) were sampled. Three trees (indicated by symbols and numbers) were sampled in each population. The geographical distances between populations are indicated.

carefully collected from a part of the branch that had not touched the ground; four acorns lying on the ground surface (on litter or mosses indicating that they were freshly fallen acorns of the year) beneath the tree crown within a distance of 2 m from the trunk; the biotic microenvironment subtending canopy acorns, defined as all tree tissues present in a cylinder of 4 cm diameter and 6 cm length around the acorn (including the acorn cupule, the cupules of other acorns, the twig to which the acorn was attached, the leaf petioles and the base of leaves); and the biotic microenvironment subtending ground acorns, defined as all substrates beneath the acorn within a cylinder of 4 cm diameter and 1 cm depth (including dead oak leaves, dead leaves of other plant species, acorn caps, twigs, pieces of bark, granules of soil, mosses, lichens or herbs). Each sample was collected aseptically, using new latex gloves and scissors cleaned with 96% ethanol to minimize contamination. Samples were stored in individual plastic vials in a cooler with ice until they could be stored at −80°C. We collected eight acorns per tree (four in the canopy and four on the ground) plus each of their biotic microenvironments, for a total of 12 mother trees located in four populations (4 populations × 3 trees × 4 sample types × 4 replicates = 192 samples). These 192 environmental samples were not surface-sterilized and are hereafter referred to as *Set#1*.

Within the same sampling campaign, we harvested 10 additional acorns that were surface-sterilized and dissected to characterize the endophytic fungal communities associated with acorn internal tissues. These acorns were harvested from five trees (two in Gèdre-Bas, two in Gabas and one in Ade). For each tree, two acorns were harvested, one in the canopy and one on the ground. We removed microbial DNA from the acorn surface by combining a classical seed disinfection protocol and a DNA decontamination protocol (Supporting Information Methods S1). After rinsing and drying the acorns on sterilized filter papers, the fruit walls and the embryos were detached using a nutcracker and pliers and stored separately at −80°C. The scissors and nutcracker were sterilized by autoclaving before dissection. Between samples, they were soaked in 70% alcohol and flame sterilized to limit cross-contamination. The autoclaved filter papers on which dissections were performed were also changed between samples. Dissections resulted in 20 samples, corresponding to 5 trees × 2 acorns × 2 tissue types (fruit wall and embryo). These 20 samples of seed internal tissues are hereafter referred to as *Set#2*.

To complement the results obtained, 16 additional *Q. petraea* acorns (*Set#3*) were collected in autumn 2017 from the ground of the oak forest of Bellebat (44°43′36.4″N, 0°13′22.5″W, southwest France) to visualize fungal colonization inside acorns using confocal microscopy. These acorns were stored at −20°C before surface-sterilization as described above, cut in half with secators and fixed overnight at 4°C in a paraformaldehyde solution (4%, w/v, in PBS, pH 7.2).

Metabarcoding of fungal communities

Samples of *Set#1* and *Set#2* were processed in December 2016 (see Methods S2 for protocol details). After DNA extraction with DNeasy Plant Mini Kits (Qiagen), metabarcoding approaches targeting the internal transcribed spacer (ITS) region of the nuclear ribosomal ITS, which is considered the universal barcode marker for fungi (Schoch *et al.*, 2012), were used to analyze the fungal communities (epiphytic and endophytic, without distinction) of *Set#1* and the endophytic communities of *Set#2*. We inferred amplicon sequence variants (ASVs) using DADA2 (Callahan *et al.*, 2016) because we showed that DADA2 outperforms clustering-based methods on a fungal mock community (Pauvert *et al.*, 2019). Positive and negative controls were used to remove contaminants, by following the method proposed by Galan *et al.* (2016) and subsequently validated using mock communities (Galan *et al.*, 2018).

Table 1 Location and description of the oak populations.

Population	Elevation asl (m)	GPS coordinates	Exposition	Average DBH (cm)	Average age (yr)
Ade	427	43°08′N, 00°00′W	South	44.82 ± 6.36	99.92
Gèdre-bas	1235	42°47′N, 00°01′E	South	43.66 ± 15.78	134.71
Bager	422	43°07′N, 00°32′W	North-west	46.73 ± 20.52	99.92
Gabas	1194	42°53′N, 00°25′W	South	40.58 ± 15.54	134.71

Average diameter at breast height (DBH) of mature trees was estimated in Vitasse *et al*. (2009). Average age was estimated by S. Delzon (pers. comm.). asl, above sea level.

SNP genotyping and maternity exclusion analyses

All acorns collected on the ground were genotyped to confirm that they originated from the tree growing above them. Genotyping was performed using 39 polymorphic single nucleotide polymorphism (SNP) markers (Gerzabek *et al.*, 2017). DNA was diluted to a final concentration of 15–20 ng μl^{-1} and sequenced using the iPLEX Gold Genotyping kit (Agena, San Diego, CA, USA) at the Genome Transcriptome Facility of Bordeaux (PGTB, Bordeaux, France) according to the manufacturer's instructions (for more details see Chancerel *et al.*, 2013). Two samples of aboveground tissues of each tree were genotyped and compared to estimate the typing error rate of false calls during genotyping. Loci with poor performance during the clustering procedure (call rates < 60%) were excluded, resulting in a final set of 28 loci. Acorn genotypes were compared to the genotype of their putative mother tree. Considering the low error rate of these SNPs (Gerzabek *et al.*, 2017), we took a deliberately conservative approach and assumed that if a given tree and acorn did not share alleles for at least one locus, the mother–offspring relationship was not confirmed. These acorns were removed from all further analyses.

Confocal microscopy

The acorns of *Set#3* were rinsed three times with PBS after fixation, immersed in 15 ml PBS containing 50 $\mu g\ ml^{-1}$ of wheat germ agglutinin (WGA)-AlexaFluor488 conjugate (Life Technologies), incubated 2 h at 37°C, and rinsed again three times with PBS. The samples were observed under a confocal microscope (Olympus Fluoview FV1000 with multiline laser FV5-LAMAR-2 and HeNe(G) laser FV10-LAHEG230-2). Pictures were taken with *x*, *y* and *z* coordinates at 405, 488 and 594 nm and with ×10, ×20, ×40 or ×60 objectives. Images were merged (RGB) using ImageJ software (Schneider *et al.*, 2012). Pictures were created using Z Project Stacks (Campisano *et al.*, 2014), cropped and the light/contrast balance was improved (Glassner *et al.*, 2015). Images presented in this publication correspond to the average colonization level observed.

Statistical analyses

Test of H1: Seed fungal communities are shaped by maternal effects and environmental filtering All statistical analyses were performed with R (R Development Core Team, 2019) using the samples of *Set#1*. To test hypothesis H1, we analyzed the effects of the mother tree (*Mother*), the local environment represented by the tree population (*Population*), and the microenvironment determined by the seed position in the canopy vs on the ground (*Seed position*) on acorn fungal community richness and composition.

Fungal richness was defined as the total number of ASVs per acorn sample and was modeled using generalized linear models (GLMs) with a negative binomial distribution and a log-link function using the Mass package v.7.3.51.4 (Venables & Ripley, 2002). The model had *Mother*, *Seed position* and their interaction nested within *Population*, and the natural logarithm of the total number of sequences per sample as an offset to take into account variation in fungal richness triggered by variation in sequencing depth (*Sequencing depth*). The offset term represents a variable with a known effect, which is commonly introduced in count-data models with a predefined coefficient (here, 1) to account for variations in sampling effort (here, sequencing depth) (Kotze *et al.*, 2012; Chiquet *et al.*, 2018), including in microbiome data models (Xia *et al.*, 2018).

Variations in fungal community composition were analyzed by using permutational multivariate analyses of variance (PERMANOVAs) with 9999 permutations, performed with the *adonis* function of the Vegan package v.2.4.6 (Oksanen *et al.*, 2019). Compositional dissimilarities among acorn samples were estimated using quantitative and binary versions of the Jaccard index (Jaccard, 1901) and visualized with principal coordinate analyses (PCoAs) using the Phyloseq package v.1.22.3 (McMurdie & Holmes, 2013). The models had *Mother*, *Seed position* and their interaction nested within *Population*, and the natural logarithm of *Sequencing depth* as the first effect. Finally, to gain insight into the mechanisms underlying the effect of *Mother* on acorn fungal community composition, we investigated whether the mother tree genotype influenced community composition in the canopy, and whether fungal community assembly in the canopy was more deterministic than in ground materials (Methods S3).

Test of H2: Similarity of seed fungal communities to the canopy environment decreases after seed fall because of horizontal acquisition of fungi from the ground To test H2, we examined the significance of the interaction between *Mother* and *Seed position* in PERMANOVA models of acorn fungal community composition. Separate models for acorns in the canopy and acorns on the ground were fitted when the interaction was significant. In addition, we investigated whether changes in fungal community composition after acorn fall were due to either the substitution of canopy-associated fungal species by ground-associated fungal species or gain of ground-associated fungal species without loss of canopy-associated fungal species, by partitioning Jaccard binary dissimilarities among acorns of the same mother tree using the Betapart package v.1.5.1 (Baselga & Orme, 2012). The proportion of fungal species of acorns on the ground also found in acorns in the canopy was calculated for each mother tree.

Test of H3: The lifestyle of fungal species influences their response to variations in the seed environment HMSCs (Ovaskainen *et al.*, 2017) were used to quantify maternal effects and environmental filtering (H1 and H2), and to test H3. In contrast to previous analyses, these models account for the biotic microenvironment of acorns (i.e. the fungal community associated with the materials surrounding each acorn) and the putative lifestyle of fungal species. They also assume that the relative effects of maternal effects and environmental filtering might differ among fungal ASVs. The models were fitted on both ASV presence–absence data and on sequence count data conditional on presence (Methods S4).

The models assumed that variation in fungal community composition among acorns (i.e. the **Y** matrix in the HMSC framework) was accounted for by four ecological predictors (*Mother mycobiota*, *Microenv mycobiota*, *Seed position* and *Site elevation*) introduced as fixed effects in the **X** matrix. The **Y** matrix represented ASV sequence counts in all acorn samples, out of which we included only the ASVs that were present in five or more acorns. *Mother mycobiota* and *Microenv mycobiota* represented fungal communities in the canopy of mother trees and in the microenvironment of acorns, respectively. They were ASV-specific predictors and thus the **X** matrix was different for every ASV of the **Y** matrix. *Mother mycobiota* was calculated for all acorn samples as the average relative abundance of the focal ASV in the twigs and leaves of mother trees, and was included at the tree level to model vertical transmission of fungi from the mother tree to its acorns. *Microenv mycobiota* was calculated as the residuals of the regression of the relative abundance of the focal ASV in the microenvironment of each acorn over its relative abundance in the canopy of the mother tree and was included at the sample level to model horizontal acquisition of fungi from the materials surrounding each acorn. *Site elevation* and *Seed position* represented filtering of fungal communities by climate and microclimate, respectively. *Site elevation* was included at the site level to model selection exerted by site-level abiotic factors, such as average air temperature, on acorn fungal communities. *Seed position* (canopy vs ground) was included at the sample level to model selection exerted by microclimate, such as higher humidity on the ground, on acorn fungal communities. *Mother mycobiota* and *Microenv mycobiota* were included in interaction with *Seed position* to test the hypothesis that their contribution to fungal communities differs between acorns in the canopy and acorns on the ground (H2). We also introduced the log-transformed sequencing depth of each sample (*Sequencing depth*) as a fixed effect in the **X** matrix, to take into account methodological biases influencing ASV sequence counts. Random effects at each hierarchical level (oak population, mother tree and seed) were also introduced to model variations in ASV sequence counts that can neither be attributed to the four ecological predictors nor to sequencing depth.

To test H3, we included available knowledge on fungal lifestyle in the **T** matrix. For each ASV, we included the trophic mode (saprotroph and/or plant pathogen) and the degree of specialization toward acorns of each ASV. The putative trophic mode of each ASV was determined using the FUNGuild database (Nguyen *et al.*, 2016) as a first approach. The information provided by FUNGuild was verified and completed by an extensive literature search. The degree of specialization of ASVs toward acorns was defined as the log-ratio of ASV relative abundance in acorns vs other sample types (i.e. branches, leaves, litter and upper soil), calculated using DESeq2 (Love *et al.*, 2014).

Test of H4: Biotic interactions at the seed scale are involved in seed protection against fungal pathogens To test H4, we interpreted the significant residual correlations among fungal ASV sequence counts at the acorn level in HMSCs as hypothetical biotic interactions among fungal strains (see Ovaskainen *et al.*, 2017). Only associations with at least 95% posterior probability were considered significant. We focused on the hypotheses of interactions among the fungal ASVs assigned at the species level and investigated whether they were known in the literature and if they corresponded to antagonistic interactions that might regulate oak pathogens.

Results

A thousand fungal ASVs were detected in and on acorns

The bioinformatic pipeline retained 21.8% of the raw MiSeq reads that corresponded to quality reads that could be assembled and assigned to a fungal phylum (Methods S2). Genotyping analysis revealed that nine of the 87 acorns collected did not originate from the putative mother tree. These samples were removed. The final ASV table was composed of 184 samples (165 of *Set#1* and 19 of *Set#2*) and 4257 fungal ASVs, representing 1891 194 sequences. The ASV table presented large variations in the total number of quality reads per sample (Table S1), with the lowest median numbers obtained for acorns in the canopy and internal tissues of surface-sterilized acorns. However, we kept all samples in the statistical analyses because they contained quality fungal reads that provide unique information on acorn fungal communities. In total, 954 fungal ASVs were detected in acorn samples.

All acorn tissues were colonized by fungi, with ascomycetes predominating

Confocal microscopy analyses of the *Set#3* samples revealed that all internal tissues from acorns collected on the ground were colonized by fungi. The fruit wall and the seed coat were the most colonized tissues, with a dense colonization under the endocarp. We also detected fungi in the embryo (Fig. 2).

Metabarcoding analyses revealed that ascomycetes dominated the fungal community. The *Set#1* samples were dominated by ascomycetes, with 91.1% and 89.4% of the whole fungal community (including both epiphytes and endophytes) of ground and canopy acorns, respectively. Dothideomycetes, Leotiomycetes and Sordariomycetes were the three main classes of ascomycetes present in the whole fungal community (Fig. S1). Several putative plant pathogens and mycoparasites were among the most abundant species (Table 2). The *Set#2* were also dominated by ascomycetes, with 79.1% and 83% of the endophytic fungal community of the seed coat and embryo, respectively (Fig. S1). The endophytic community of acorns differed from the fungal community as a whole because of the higher proportion of Microbotryomycetes (Fig. S1; Table S2), a class of basidiomycetes containing endophytes (also known as anther smut fungi) that manipulate plant reproduction during their pathogenic stage (Antonovics *et al.*, 2018).

Seed fungal communities were shaped by maternal effects and environmental filtering, with maternal effects predominating (H1)

In acorns that were not surface-sterilized (*Set#1*), fungal community richness (Table 3) and composition (Table 4; Fig. 3) differed

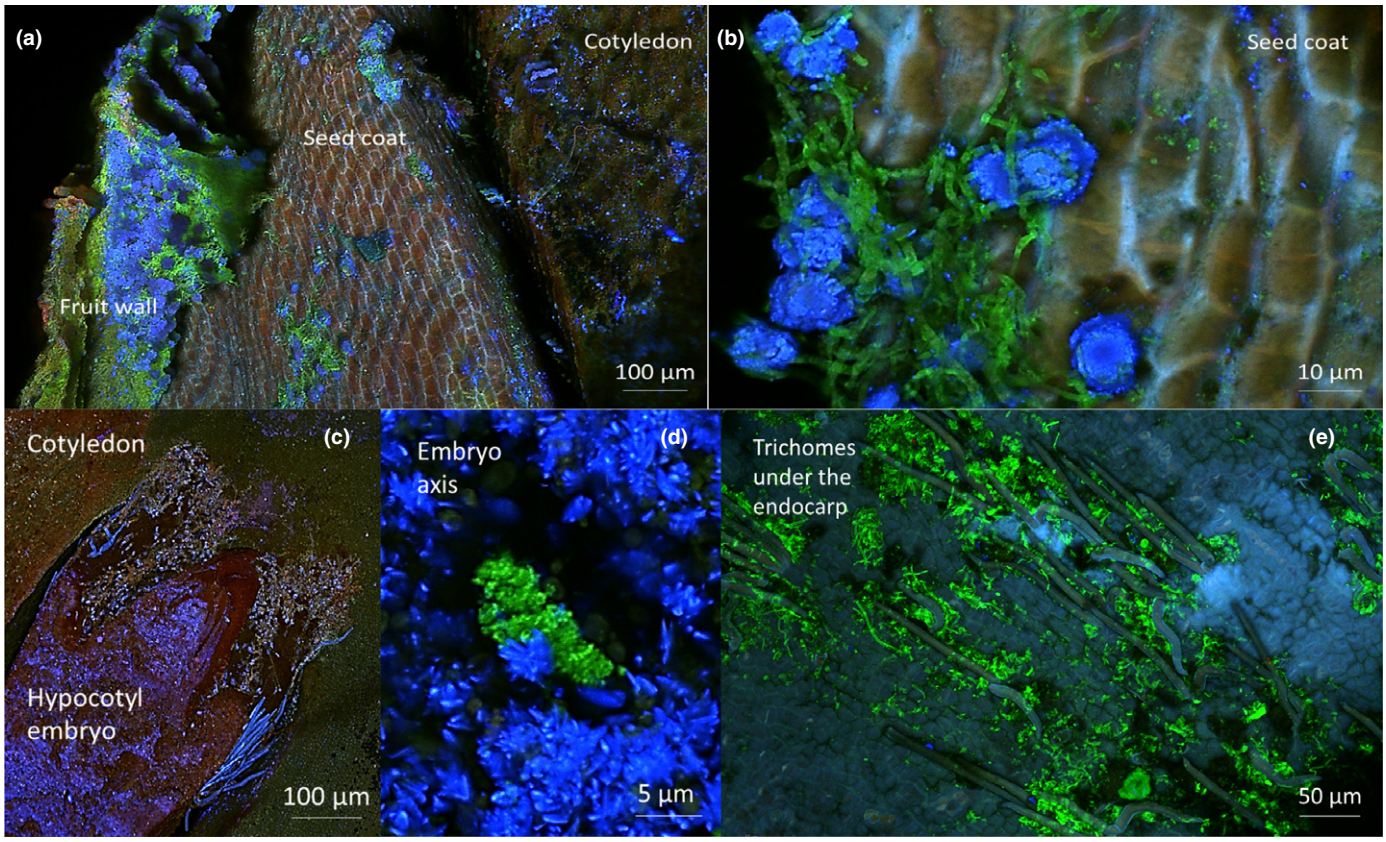

Fig. 2 Endophytic fungi in cross-sections of acorns of sessile oak collected on the ground and surface-sterilized. Fungi (green fluorescent) were revealed by confocal microscopy and WGA-ALEXA fluor488 staining. (a) Fruit wall and seed coat. (b) Zoomed-in view of (a). (c) Embryo and cotyledon. (d) Zoomed-in view of (c). (e) Internal surface of the endocarp.

significantly among oak populations and mother trees within the same population, confirming the hypothesis that both maternal effects and environmental filtering shape acorn fungal communities (H1). When the qualitative Jaccard index was used to assess compositional dissimilarity among samples, the mother tree explained a larger part of variance in community composition than the population (11.6% vs 8.7%; Table 4), suggesting that maternal effects better explained fungal ASV presence–absence than environmental filtering. These results were consistent with those of the HMSC model of ASV presence–absence (Table 5). According to this model, elevation was a minor direct driver of fungal community composition (2% of the explained variance). By contrast, the average relative abundance of a fungal ASV in the tissues of a mother tree (*Mother mycobiota*) was the second most important predictor of the occurrence of this ASV in an acorn from this tree, in interaction with the acorn position (39% of the explained variance). *Mother mycobiota* was the unique predictor of occurrence for several ASVs belonging to the orders Helotiales, Venturiales and Xylariales (Fig. 4).

Genetic distance among mother trees did not account for variations in fungal community composition in acorns, twigs or leaves (Table S3). However, community assembly was significantly less stochastic in twigs and leaves than in ground materials (Table S4), suggesting that selective forces are exerted on aboveground fungal communities by mother trees. Community stochasticity level, measured with the normalized stochasticity ratio (NST; Ning *et al.*, 2019), increased from mother tree aboveground tissues (53% on average), to acorns in the canopy (70.05%) and then to acorns on the ground (80.45%) and ground materials (80.95%) (Table S4).

Similarity of seed fungal communities with the canopy environment decreased after seed fall because of horizontal acquisition of fungi from the ground (H2)

In acorns that were not surface-sterilized (*Set#1*), the fungal community differed significantly in richness (Table 3) and composition (Table 4) between acorns in the canopy and on the ground. Fungal richness increased and composition shifted toward that of ground materials after acorn fall (Fig. 5; Table S5). Acorns on the ground shared a higher proportion of fungal ASVs with ground materials than acorns in the canopy (65% vs 56% on average, Table S5), confirming the horizontal acquisition of fungi from the ground. Turnover accounted on average for 75% of Jaccard beta-diversity (Table S6), indicating that the horizontally acquired fungi replaced those originating from the canopy rather than adding to the community, in accordance with our hypothesis (H2). The HMSC presence–absence model confirmed the large influence of horizontal acquisition on acorn fungal communities (Table 5). The relative abundance of a fungal ASV in the

Table 2 Most abundant fungal species associated with seeds of sessile oak and their microenvironment.

Fungal species name and phylum (CL > 80%)	Average relative abundance per sample type (%)					Lifestyle	Reference(s)
	All seeds ($n = 78$)	Seeds in the canopy ($n = 40$)	Canopy samples (twigs, leaves) ($n = 40$)	Seeds on the ground ($n = 38$)	Ground samples (litter, upper soil) ($n = 47$)		
Gnomoniopsis paraclavulata (A)	13.4	5.3	1.5	21.9	0	Pathogen isolated in leaves, buds, cupules and shoots of *Castanea sativa*. Commonly isolated from overwintered leaves of *Quercus* sp.	Tosi *et al*. (2015), Sogonov *et al*. (2008)
Stromatoseptoria castaneicola (A)	2.7	5.2	0.9	0	0.2	Causes leaf spots on *Castenea sativa*	Quaedvlieg *et al*. (2013)
Taphrina carpini (A)	2.4	3.4	0.7	1.4	0.3	Common pathogen encountered on Fagaceae leaves	Bacigálová (1991), Inácio *et al*. (2004), Cordier *et al*. (2012b)
Epicoccum nigrum (A)	2.3	0.4	0.1	4.2	0.2	Ubiquitous fungus found in soil, leaves and seeds described as primary saprotroph and plant pathogen	Ahumada-Rudolph *et al*. (2014)
Mycosphaerella tassiana (A)	1.7	1.5	0.1	1.9	0.3	Common pathogen found in the phyllosphere including that of oak	Schubert *et al*. (2007), Jakuschkin *et al*. (2016)
Curvibasidium cygneicollum (B)	1.5	1.2	0.2	1.9	0.1	Endophyte of fruits, leaves, trunk and soil behaving as a phytopathogen or a mycoparasite. The species is insensitive to the mycocins produced by *Filobasidium* and *Cystofilobasidium*	Sampaio *et al*. (2004), Mašínová *et al*. (2017)
Cylindrium elongatum (A)	1.5	1.4	0.1	1.5	4.3	Bacterial and fungal antagonist found on oak leaves	Reyes-Estebanez (2011), Duarte *et al*. (2015)
Polyscytalum algarvense (A)	1.5	1.9	0	1	0	Necrotroph fungi found on *Eucalyptus* leaves	Cheewangkoon *et al*. (2009), Crous *et al*. (2018)
Fusarium pseudensiforme (A)	1.3	1.4	0	1.2	0	Found on bark of trees	Nalim *et al*. (2011)
Cladosporium delicatulum (A)	1.3	1.3	0.2	1.3	0.3	Found in cereal seeds, mycoparasite of *Taphrina* spp. and *Magnaporthe oryzae*	Amanelah Baharvandi & Zafari (2015), Chaibub *et al*. (2016)

Seeds were either collected in the tree canopy or on the ground. Materials from the seed microenvironment (twigs and leaves, or litter and upper soil) were also collected. The fungal community of all four sample types was analyzed using a metabarcoding approach. Only amplicon sequence variants (ASVs) assigned to Ascomycota (A) or Basidiomycota (B) with the UNITE database were kept. Average relative abundances of all ASVs were computed for each sample type, after merging ASVs assigned to the same fungal species. Only ASVs assigned to species with a confidence level (CL) > 80% are shown in the table.

microenvironment of an acorn (*Microenv mycobiota*), in interaction with the acorn position, was generally the best predictor of ASV occurrence (47% of the explained variance), especially for the orders Capnodiales, Dothideales and Taphrinales (Fig. 4a).

Despite the turnover in fungal community composition after acorn fall, some effects of the mother on acorn fungal communities persisted after acorn fall. The statistical interaction between the mother tree and the seed position was not significant for fungal richness (Table 3) or community composition measured with the qualitative Jaccard index (Table 4), indicating that mother tree effects in ASV richness and occurrence were similar for ground acorns and canopy acorns. The analysis of fungal ASVs shared between ground and canopy acorns (Table S5) confirmed that some maternal species were retained after acorn fall. Overall, acorns on the ground shared 10–40% of their fungal community with acorns in the canopy of the same mother tree, and 21–50% with mother tree leaves and twigs. On average, 38% of fungal ASVs of acorns on the ground were present in both acorns in the canopy and mother tree tissues. Fungal species most often retained after acorn fall were *Taphrina* sp., *Cladosporium delicatulum*, a mycoparasite of *Taphrina* sp. (Baharvandi & Zafari, 2015) and the ubiquitous *Epicoccum nigrum*. In contrast to the mother tree effects in ASV richness and occurrence, the mother tree effects in ASV relative abundance disappeared after acorn fall. The interaction between the mother tree and the seed position was significant for community composition measured with the quantitative Jaccard index (Table S7) and separate analyses on canopy and ground acorns revealed that mother tree effects were only significant for canopy acorns (Table S7).

Response of seed fungal species to elevation and seed fall depended on their lifestyle (H3)

The HMSCs showed that fungal lifestyle influenced the response of fungal ASVs to environmental variation, in accordance with our hypothesis (H3). High elevation selected for saprotroph

species and seed specialists, increasing their proportion in acorn fungal communities (Table S8). Acorn fall favored pathogen species and saprotrophs. Their proportion increased after acorn fall whereas their abundance (conditional on presence) was not altered (Table S8). These findings suggest that vertically transmitted pathogens did not increase in abundance after acorn fall and that acorns were colonized by both pathogen and saprotroph species from the ground.

Table 3 Generalized linear model (GLM) of fungal community richness of seeds of sessile oak.

	df	χ^2	*P*-value
Population	3	20.765	<0.001
Mother (Population)	7	14.173	<0.05
Seed position (Population)	4	23.678	<0.001
Mother × Seed position (Population)	7	11.121	0.13

Fungal richness was defined as the total number of amplicon sequence variants (ASVs) per seed sample. The model had *Mother*, *Seed position* and their interaction nested within *Population*, and the natural logarithm of the total number of sequences per sample as an offset.

Table 4 Permutational multivariate analysis of variance (PERMANOVA) of compositional dissimilarities among fungal communities of seeds of sessile oak.

	df	SS	MS	*F*-value	*P*-value	R^2
Sequencing depth	1	0.551	0.550	1.35	<0.01	0.015
Population	3	3.026	1.008	2.47	<0.001	0.087
Mother (Population)	8	4.060	0.507	1.24	<0.001	0.116
Seed position (Population)	4	2.240	0.559	1.37	<0.001	0.064
Mother × Seed position (Population)	7	2.844	0.406	0.99	0.52	0.081
Residuals	54	22.019	0.407			0.633
Total	77	34.739				1.000

Dissimilarities among seed fungal communities were estimated using the Jaccard binary distance. The model had the mother tree (*Mother*), the seed position (*Seed position*, in the canopy or on the ground) and their interaction nested within the tree population (*Population*), and the natural logarithm of *Sequencing depth* as the first effect. df, degrees of freedom, SS, sum of squares, MS, mean sum of squares.

Fungal pathogens were associated with their mycoparasites in the seed (H4)

The confocal microscopy analyses of *Set#3* samples had shown that fungal colonization was very dense on internal surfaces of the fruit wall of fallen acorns (Fig. 2e), indicating that fungal colonizers might enter into contact and compete for space and eventually resources. We expected such competitive interactions to generate negative associations among ASV sequence counts. In contrast to this expectation, residual co-occurrence patterns of the HMSCs

Table 5 Partitioning of the variance in fungal community composition of seeds of sessile oak.

Predictor			Explained variance (%)	
Type	Level	Name	PA model	SC model
Fixed	Seed	*Sequencing depth*	1	17
Fixed	Seed	*Seed position (P)*	4	8
Fixed	Seed	*Microenv mycobiota × P*	47	23
Fixed	Tree	*Mother mycobiota × P*	39	25
Fixed	Population	*Elevation*	2	6
Random	Seed	*Seed*	3	9
Random	Tree	*Tree*	1	6
Random	Population	*Population*	2	6

Four fixed effects were included in the HMSCs to explain variations in the presence–absence (PA) or the sequence count (SC) of a focal fungal amplicon sequence variant (ASV) among seeds: *Sequencing depth* (total number of sequences per sample), *Seed position* (canopy or ground), *Microenv mycobiota* (relative abundance of the focal ASV in the seed biotic microenvironment), *Mother mycobiota* (average relative abundance of the focal ASV in the mother tree aboveground tissues), and *Elevation*. Random effects were included at each spatial scale (seed, tree and population). Results of variance partitioning are given as percentages (%) of the total explained variance.

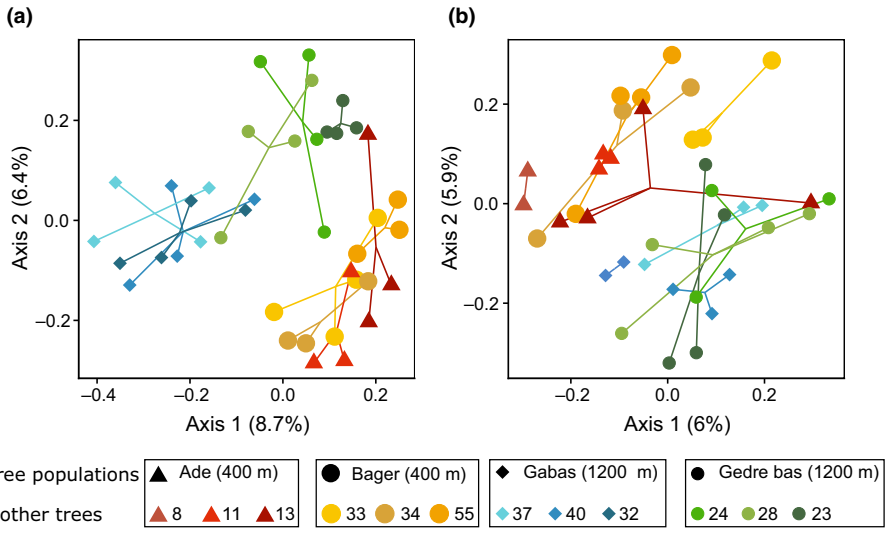

Fig. 3 Compositional dissimilarities among fungal communities of seeds collected (a) in the canopy and (b) on the ground in four populations of sessile oak. Dissimilarities among seeds were estimated using binary Jaccard distance and represented with a PCoA plot. Fungal community composition differed significantly among tree populations and among mother trees (Table 4). Seeds belonging to the same mother tree were represented with the same color and connected with lines to their group centroid.

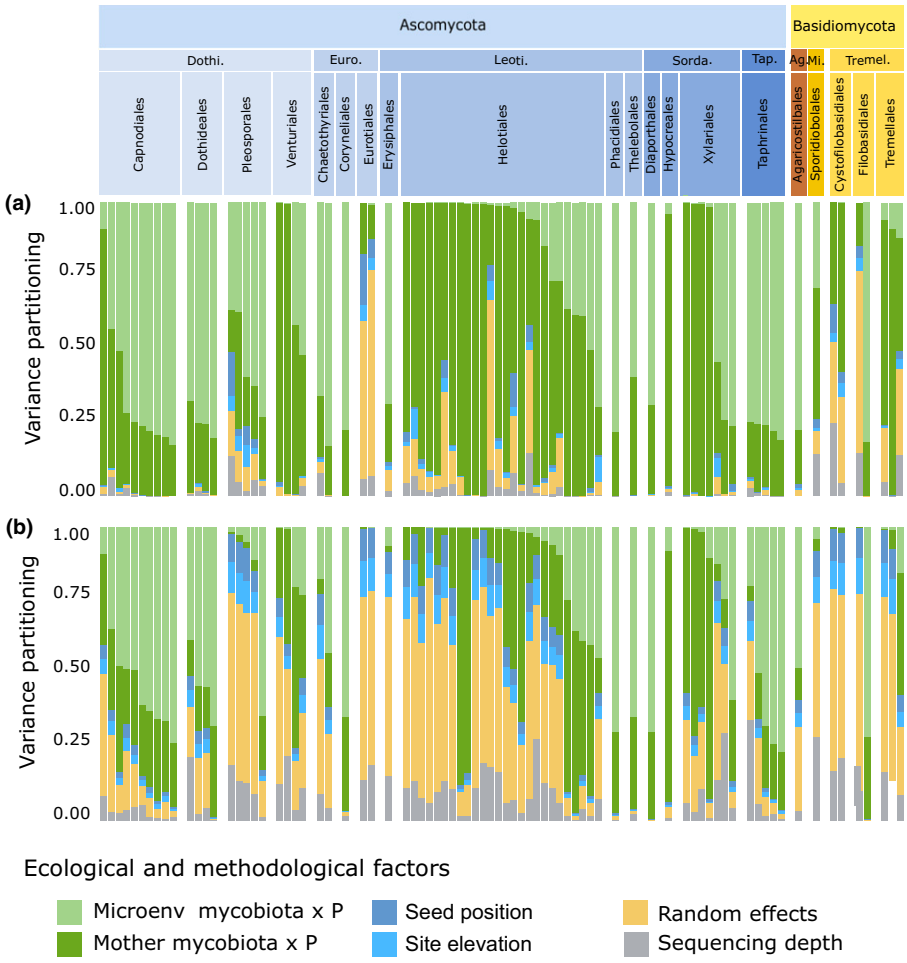

Fig. 4 Partitioning of the variance in the composition of seed fungal communities of sessile oak. Four fixed effects were included in the HMSCs to explain variations in (a) the presence–absence or (b) the sequence count of a focal fungal amplicon sequence variant (ASV) among seed samples: *Sequencing depth* (total number of sequences per sample), *Seed position* (*P*, canopy or ground), *Microenv mycobiota* (relative abundance of the focal ASV in the seed microenvironment), *Mother mycobiota* (relative abundance of the focal ASV in the mother tree aboveground tissues) and *Site elevation*. *Mother mycobiota* and *Microenv mycobiota* were introduced in interaction with *Seed position* (*P*). Random effects were included at each spatial scale (seed, tree and population). Results of variance partitioning are given as percentages (%) of the total explained variance. ASVs are ranked by fungal phylum, class and order (Dothi, Dothideomycetes; Euro, Eurotiomycetes; Leo, Leotiomycetes; Sorda, Sordariomycetes; Tap, Taphrinomycetes; Ag, Agaricostilbmoyecetes; Mi, Microbotryomycetes; Tremel, Tremellomycetes). Only ASVs assigned at the order level are shown.

at the acorn level revealed only positive associations between fungal ASVs. Among the associated fungal ASVs, 14 could be assigned at the species level (Fig. 6). Six of them are described as plant pathogens and two of them, *Mycosphaerella tassiana* and *Taphrina carpini*, have already been found in association with oak (Table S9). Four species are described as mycoparasites, including *Cladosporium delicatulum*. The positive association between *T. carpini* and *C. delicatulum* (Fig. 6), which were found both in the embryo and in the fruit wall (Table S2), might therefore represent a vertically transmitted host–parasite interaction (i.e. between a fungal pathogen and its mycoparasite). These findings suggest that biotic interactions structure fungal communities of acorns and might play a role in oak disease regulation, in accordance with our hypothesis (H4).

Discussion

The seed mycobiome of trees has received little attention despite its probable role in forest regeneration and resilience to biotic and abiotic stresses (Vivas *et al.*, 2015, 2020). To fill this gap, we analyzed fungal communities of seeds in four unmanaged populations of sessile oak (*Q. petraea*) and showed that the fungal mycobiome varied among mother trees and environments.

Seed mycobiome composition and its influence on forest health

Our confocal microscopy analyses revealed a dense fungal colonization on internal surfaces of fruit walls of fallen acorns and confirmed the presence of fungi within embryos of *Q. petraea*. The endophytic fungal communities of oak seeds contained ubiquitous fungal species, such as *E. nigrum* (Andrews & Harris, 2000), and endophytic yeasts previously described in the reproductive organs of other plant species, such as *Curvibasidium cygneicollum* (Sampaio *et al.*, 2004; Mašínová *et al.*, 2017; Antonovics *et al.*, 2018). Seeds also contained fungal pathogens and antagonists of the pathogens. For instance, we detected two foliar pathogens of Fagaceae tree species, *M. tassiana* and *T. carpini* (Bacigálová, 1991; Schubert *et al.*, 2007), and a mycoparasite of *Taphrina* species, *C. delicatulum* (Baharvandi & Zafari, 2015; Chaibub *et al.*, 2016). Our network analyses revealed a positive association between *T. carpini* and *C. delicatulum*, in line with the mycoparasitic interaction described in the literature between these two species. Both the fungal pathogen and its mycoparasite were detected as seed endophytes, suggesting that mother trees not only transmit fungal pathogens to their progeny but also antagonists of these pathogens.

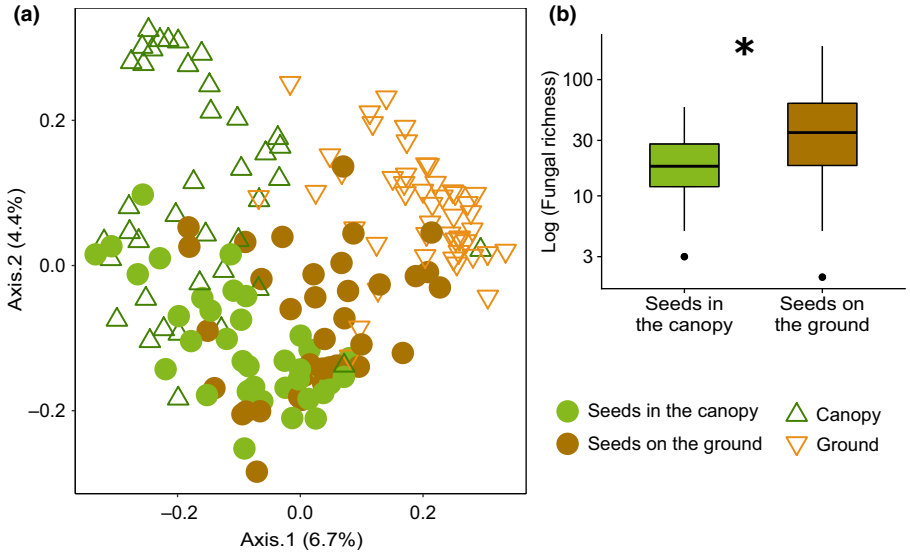

Fig. 5 Fungal community composition and richness of seeds of sessile oak collected in the canopy and on the ground. (a) PCoA plot of compositional dissimilarities among fungal communities associated with seeds, canopy (leaves and twigs) and ground materials (litter and upper soil). Dissimilarities among samples were estimated using binary Jaccard distance. Fungal community composition differed significantly among the four sample types (PERMANOVA on Jaccard qualitative index; $F = 4.8$, $P < 0.001$; PERMANOVA on Jaccard quantitative index; $F = 4.3$, $P < 0.001$). (b) Richness (log-transformed) of seed fungal communities, defined as the number of amplicon sequence variants (ASVs) per sample. The lower and upper edges of each box correspond to the 25th and 75th percentiles, respectively, while the whiskers correspond to the 10th and 90th percentiles. Dots indicate outliers. A horizontal line indicates the median value. Richness was significantly higher (*) in seeds on the ground according to GLM results (Table 3).

These findings could have important implications for tree breeding and seed production. It would be interesting to identify which maternal genes and environments favor the vertical transmission of mycoparasites, to produce seeds that contain these natural biocontrol agents and thereby foster the resistance and resilience of planted forests to fungal diseases. Such selective breeding might have more sustainable effects than the direct application of mycoparasites (e.g. in cacao orchards; Krauss *et al.*, 2010, 2013) and could be useful for larger, wood-producing plantations.

Maternal effects in seed mycobiome composition and forest adaptation to environmental change

A major result of our study is that seed fungal communities differed significantly among trees within the same oak population. Our study uniquely demonstrates, using two statistical approaches, that the mother tree influences seed mycobiome composition, which is a component of the offspring community phenotype (Whitham *et al.*, 2006). Such influence can be considered a maternal effect (*sensu* Wolf & Wade, 2009). Both approaches revealed a major effect of the mother on the identity of the fungal species associated with seeds. The PERMANOVA model accounted for 36.7% of the variance in fungal ASV presence–absence, with the mother tree accounting for the largest part of the explained variance (31.6%). HMSC accounted for 80% of the variance in ASV presence–absence, with the average fungal community composition in the crown of the mother tree directly accounting for 39% of the explained variance. In our study, the mother tree effect was partially due to selection of fungal species in aboveground mother tree tissues. Twigs and leaves harbored the most deterministic fungal communities, in comparison to seeds and ground materials. Community assembly was less deterministic in seeds than in twigs and leaves, but it was not purely stochastic, suggesting that seed fungal communities assembled from a set of fungal species selected by the mother that were then shaped by stochastic colonization events. These events might have increased divergence in seed fungal community composition among mother trees through priority effects (Fukami, 2015). It would be interesting to tease apart the mother tree genetic effect, by investigating whether aboveground fungal communities were genetically determined (Cordier *et al.*, 2012a; Hunter *et al.*, 2015; Sapkota *et al.*, 2015). We did not find support for a genetic effect in this study, but our analyses were only based on a few neutral genetic markers. Future studies should consider additional markers. Another interesting next step would be to investigate whether the maternal effects continue into the seedling stage, as found by Vivas *et al.* (2017) on *Eucalyptus* seedlings.

Our analyses also indicated a significant, but weak, effect of environmental filtering on seed fungal communities in *Q. petraea*. We found that the richness and composition of seed fungal communities varied significantly among the four oak populations, which were located in two watersheds, with within-watershed locations separated by up to 800 m in elevation. However, the HMSCs revealed that the direct influence of elevation on the seed mycobiome was small. Elevation accounted for only 2% of the explained variance in patterns of fungal ASV presence–absence, and 6% of the variance in ASV abundance. By contrast, the fungal community composition in the materials directly

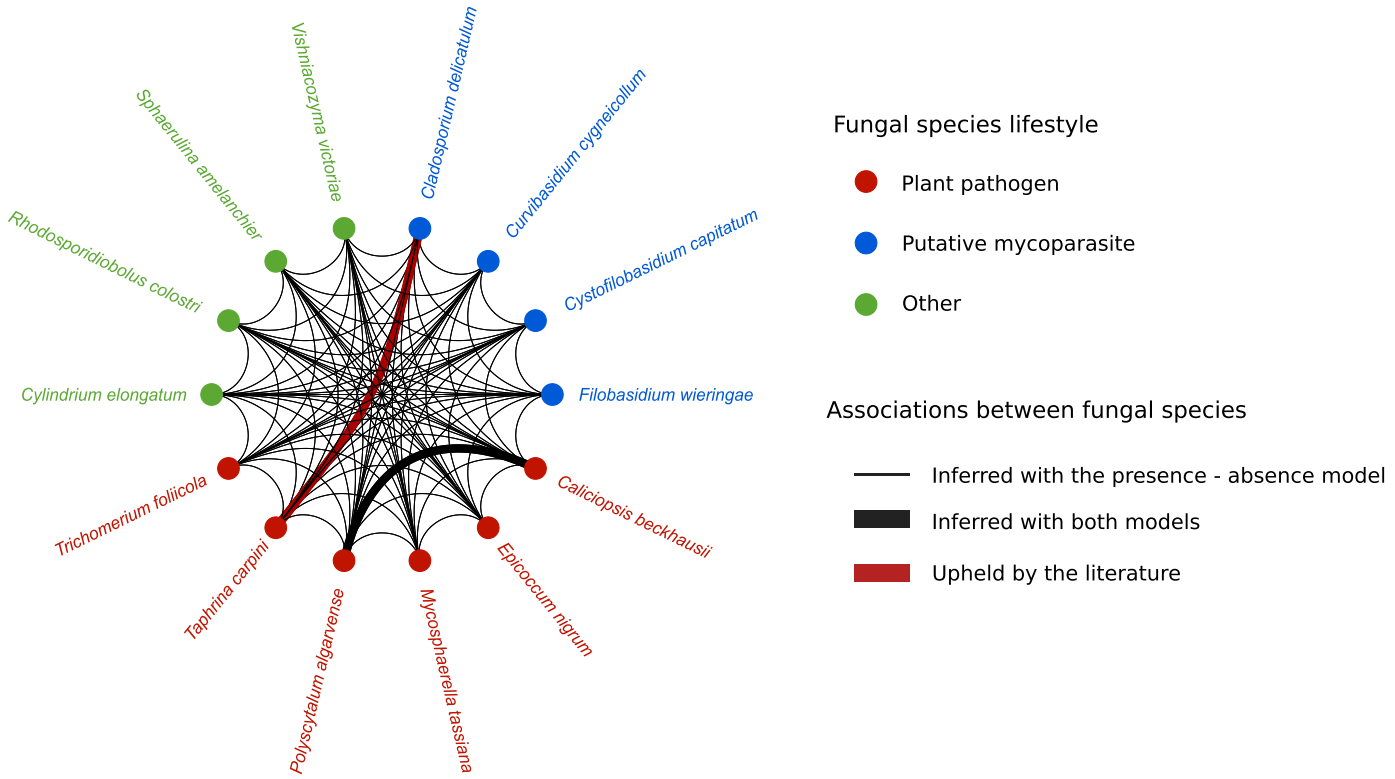

Fig. 6 Network of fungal associations estimated by HMSCs. Associations were all positive. Network nodes correspond to fungal amplicon sequence variants (ASVs) assigned to the species level and their color corresponds to putative functions according to FUNGuild and the literature (Supporting Information Table S9). Network links indicate associations with at least 95% posterior probability estimated by the presence–absence model (thin black plain line) or by both the presence–absence model and the sequence count model (thick black plain line). The positive association between *Taphrina carpini* and *Cladosporium delicatulum* is indicated in red because it is described in the literature as a host–parasite interaction (Table S9).

surrounding the seed (on the mother: leaves and twigs; on the soil: litter and upper soil) accounted for 47% and 23% of the explained variance, respectively. These findings suggest that the biotic microenvironments surrounding seeds, including mother tree tissues, are stronger drivers of seed mycobiome assembly than environmental filtering by abiotic conditions. However, the analysis of a larger number of sites along elevation gradients would be needed to confirm the weak direct effect of elevation on the seed mycobiome.

Numerous studies have shown that the fungal communities thriving in the aboveground parts (leaves and twigs) of forest trees vary in composition along elevation gradients, including the gradient of the present study (Cordier *et al.*, 2012b; Coince *et al.*, 2014; Vacher *et al.*, 2016). Our results suggest that some of these locally adapted fungal species can be transmitted by mother trees to seeds, but that not all mother trees transmit all fungal species. Each mother tree appears to exert a strong additional selection on the seed mycobiome, which could determine the mother tree's own fitness by influencing seedling growth and survival. At the tree population level, these variations in mycobiome transmission could increase the phenotypic variability in the offspring and favor population adaptation to environmental change (Vannier *et al.*, 2015; Vujanovic *et al.*, 2019). In turn, the selection of mother trees transmitting the most beneficial mycobiome could shape the local fungal community, linking the evolution of tree populations with the dynamics of forest biodiversity. However, studies of plant species with shorter generation times than oaks will be needed to experimentally confirm this eco-evolutionary scenario (Post & Palkovacs, 2009).

Seed mycobiome transition from canopy to soil and forest regeneration

Finally, our analyses showed that seed fall corresponds to a major transition in the seed mycobiome of sessile oak. Fungal community richness significantly increased and composition shifted toward that of ground materials after seed fall, confirming that seeds on the ground are rapidly colonized by the fungal species present in the surrounding microenvironment (Truyens *et al.*, 2015; Klaedtke *et al.*, 2016; Qin *et al.*, 2016). For instance, *Gnomoniopsis paraclavulata*, which was previously found in association with oak litter (U'Ren & Arnold, 2016), drastically increased in abundance after seed fall. Our analyses also suggested that seed fall triggers a replacement of canopy-inherited species by ground-derived species, rather than an addition of species associated with ground materials. However, this replacement was only partial, as 35% of the fungal ASV on fallen seeds were on average also found in the mother tree aboveground tissues, explaining why some maternal effects in seed mycobiome composition persisted after seed fall.

To better understand the processes driving the success of forest regeneration, future research will have to analyze the network of microbial interactions that builds up at the aboveground–belowground interface after seed fall. Such studies should experimentally test if maternally transmitted species shape these networks through priority effects (Fukami, 2015) and link network dynamics with seed and seedling health and survival. The influence of vertically transmitted fungal species on seed dispersal could also be investigated, as previous research on oak trees showed a significant relationship between a fungus-like pathogen associated with seeds and the abundance of several oak-dependent bird species, including seed dispersers (Monahan & Koenig, 2006).

Conclusions

Our study revealed that diversified fungal communities colonize seeds of sessile oak, including their internal tissues. For what we believe is the first recorded observance in *Q. petraea*, we found fungi in the embryo. The seed fungal communities were shaped by maternal effects, environmental filtering and biotic interactions. Maternal effects were stronger drivers of seed mycobiome composition than environmental filtering by abiotic conditions. Biotic interactions included several host–parasite interactions between fungal pathogens and mycoparasites, one of which was likely to be vertically transmitted. Such vertical transmission of biotic interactions from the mother tree to its progeny through seeds could have important implications for forest health, dynamics and evolution. Future experiments should analyze: how the maternal genes and the environment interact to determine the identity and function of vertically transmitted fungi; if these vertically transmitted fungal species influence, through priority effects, the dynamics of microbial networks developing during seed germination; and the relationship between network dynamics and seedling health. This research will allow us to integrate seed microbial ecology into programs of tree breeding and seed production, as well as predictive models of forest dynamics and evolution.

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Author contributions

CV, AEZ and SD designed the research; TF, TC and CV conducted fieldwork; TF, CP and SC performed experiments; TF, CP, OO and CV analyzed and interpreted the data; MB and AH contributed with protocols and analysis tools; TF and CV wrote the manuscript, with input from all authors.

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Data availability

All raw sequences obtained from the sequencing of seeds of sessile oak and their biotic microenvironment are available from the National Center for Biotechnology Information Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA551388. The code, ASV tables and metadata (including ASV richness, sequencing depth, site and sample descriptions, and genotypic data) are available as an archive at https://doi.org/10.15454/0CNFWS.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Taxonomic composition of fungal communities of seeds of sessile oak.

Methods S1 Protocol used to remove microbial DNA from the seed surface.

Methods S2 Protocols and pipelines used for the metabarcoding of fungal communities.

Methods S3 Methods used to investigate the mechanisms underlying maternal effects.

Methods S4 Methods used to fit the HMSCs.

Table S1 Number of fungal reads per sample after all quality filters.

Table S2 Most abundant fungal species found as endophytes in seeds of sessile oak.

Table S3 Mantel correlations between the genetic distance among mother trees and compositional dissimilarities among fungal communities.

Table S4 Contribution of stochastic processes to community assembly.

Table S5 Number and percentage of fungal ASVs of seeds shared with ground materials and canopy tissues.

Table S6 Nestedness and turnover components of compositional dissimilarities among seed fungal communities.

Table S7 PERMANOVAs of variance of compositional dissimilarities among seed fungal communities.

Table S8 Response of fungal ASVs to site elevation and seed position depending on their lifestyle according to HMSCs.

Table S9 Fungal ASVs positively associated with another ASV at the seed level according to HMSCs.

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