



Experimental evidence of microbial inheritance in plants and transmission routes from seed to phyllosphere and root

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Summary

While the environment is considered the primary origin of the plant microbiome, the potential role of seeds as a source of transmitting microorganisms has not received much attention. Here we tested the hypothesis that the plant microbiome is partially inherited through vertical transmission. An experimental culturing device was constructed to grow oak seedlings in a microbe-free environment while keeping belowground and aboveground tissues separated. The microbial communities associated with the acorn's embryo and pericarp and the developing seedling's phyllosphere and root systems were analysed using amplicon sequencing of fungal ITS and bacterial 16S rDNA. Results showed that the seed microbiome is diverse and non-randomly distributed within an acorn. The microbial composition of the phyllosphere was diverse and strongly resembled the composition found in the embryo, whereas the roots and pericarp each had a less diverse and distinct microbial community. Our findings demonstrate a high level of microbial diversity and spatial

partitioning of the fungal and bacterial community within both seed and seedling, indicating inheritance, niche differentiation and divergent transmission routes for the establishment of root and phyllosphere communities.

Introduction

All plant species, without exception, have been found to be associated with a diverse community of microbes. The first known association between two microorganisms occurred around 1.45 billion years ago, between a prokaryote now known as a mitochondrion and an archaeon, which led to the creation of eukaryotes (Roberts, 2017). A billion years later, the merging of a eukaryote with a photosynthetic bacterium (now known as a plastid) represented the first step of plant evolution (Sagan, 1967; Gray, 2017). In current ecological systems, plants have been shown to recruit, actively or passively, microbes that form a dynamic assemblage of species referred to as the plant microbiome. The fossil record indicates that plants have been associated with endophytic fungi and bacteria for more than 400 million years (Krings *et al.*, 2007). Plants pass vital endosymbionts such as mitochondria and chloroplasts to their offspring through cytoplasmic inheritance; however, data on the inheritance and continuity of a distinct plant microbiome, and how the transfer occurs, are lacking. This raises a fundamental question, where do plant-associated microbes come from? Thus far, the microbial community associated with different parts of a plant, including seeds, has been shown to be mainly influenced by plant species, soil, climatic factors and management practices in the case of agricultural crops (Chaparro *et al.*, 2012; Adam *et al.*, 2018). The environment, soil in particular, is widely considered as the origin and main source of microorganisms (Gopal and Gupta, 2016; Yeoh *et al.*, 2017). Some have argued that similarities in the microbial composition between plants of the same species are largely due to selective recruitment from the surrounding environment and are governed by processes related to the physiological, morphological and genetic traits of the plant species

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(Bouffaud *et al.*, 2014; Yeoh *et al.*, 2017; Hassani *et al.*, 2018). This explanation is only valid, however, if all environments contain all (or most) microorganisms required for the assembly of the plant microbiome. Additionally, the argument ignores the potential contribution of seeds to the assembly of the plant microbiome, which would explain similarities in the microbiome of a plant, irrespective of the local source pool. While information on the seed microbiome is sparse relative to data on the rhizosphere and phyllosphere, the studies that have been conducted have reported contradictory results. Some studies indicate that surface sterilization was enough to obtain microbe-free seeds (Lundberg *et al.*, 2012; Bai *et al.*, 2015), while others found a very limited number of microbes (Hodgson *et al.*, 2014; Newcombe *et al.*, 2018) to high microbial diversity including fungi, bacteria and archaea (Compant *et al.*, 2011; F rnkranz *et al.*, 2012; Xu *et al.*, 2014; Solanki *et al.*, 2019; Wassermann *et al.*, 2019; Matsumoto *et al.*, 2021) retained in surface-sterilized seeds.

Regardless of the number of potential species, different seed tissues (e.g. seed coat, embryo, endosperm and perisperm) have been reported to each harbour distinct microbial communities (Singh and Mathur, 2004; Shade *et al.*, 2017). A proposed categorization of seed-associated microbes into (i) transient - found in seed but not necessarily transmitted to the developing seedling and (ii) persistent or transmitted - found in seeds and transmitted to the developing seedling, has also been suggested (Shade *et al.*, 2017). The idea of vertical transmission, the direct transfer of a microorganism from a parent to its progeny, is a universal phenomenon in animals (Funkhouser and Bordenstein, 2013; Berg and Raaijmakers, 2018). Since seeds are considered the plant progeny, the majority of studies on vertical transmission in plants have focused on the composition and assembly of the seed's microbial community, rather than the dynamics of establishment and transmission during seedling development, despite the latter being a crucial step in maintaining the continuity of the plant microbiome and a potential bottleneck in vertical transmission (Gundel *et al.*, 2011; Hodgson *et al.*, 2014; Bergna *et al.*, 2018; Shahzad *et al.*, 2018; Hardoim, 2019; Rochefort *et al.*, 2019; Wassermann *et al.*, 2019; Rodr guez *et al.*, 2020) (Fig. 1A). Hodgson *et al.* (2014) identified two fungal species in pollen, seed and leaves of forbs grown in sterile conditions (Hodgson *et al.*, 2014). Walitang *et al.* (2019) reported several bacterial endophytes to be present in both rice seed and leaves (Walitang *et al.*, 2019). Although these studies indicate potential vertical transmission via the seed, the similarity between the microbial communities found in seeds and plant tissue does not unequivocally demonstrate microbial inheritance. Demonstrating inheritance

under natural conditions is problematic as plants are exposed to and dependent on their surrounding environment, especially the soil with its high microbial diversity, which makes distinguishing between vertical and horizontal transmission a challenging endeavour.

In the current study, we developed and used a novel culturing device to grow common oak seedlings in microbe-free conditions while keeping the below- and aboveground plant parts separated. The use of this device allowed us to characterize the composition and subsequent dynamics of the seed microbiome, and also overcome the problem of discerning the source of microbial recruitment. An amplicon-based approach of the ITS and 16S regions was used to characterize the fungal and bacterial community present in the embryo and pericarp of acorns, as well as the roots and phyllosphere of the developing seedling. This approach was used to answer the following questions:

- i. What is the diversity and composition of the microbial community in oak acorns (inherited microbiome) and how are these microbes distributed between embryo and pericarp?
- ii. Is the seed microbiome transient or transmitted to the developing seedling?
- iii. If there is a transmitted microbiome, how is it distributed between the roots and phyllosphere during seedling development?

We defined the transient microbiome as the microbial taxa present in the seed that are inherited from the parent plant but are not passed on to the developing plant (pink colour in Fig. 1B). The transient microbiome in the present study represents the microbial taxa present in either the embryo, pericarp, or both, but not in the phyllosphere or roots of the germinated seedling. The transmitted microbiome represents taxa that are vertically transmitted to the phyllosphere or roots of the developing seedling (green colour in Fig. 1B). The transmitted microbiome in the current study is represented by taxa shared between an acorn (embryo and pericarp) and the phyllosphere, roots, or both in the developing seedling.

We expected to find a high microbial diversity in acorns, with a higher diversity in the pericarp than in the embryo, as previous studies have shown that embryonic tissues harbour few bacteria compared with outer seed layers (Glassner *et al.*, 2018). Given the structural and chemical differences that exist between seed embryo and pericarp, we expected to find spatial compartmentalization of the microbial community between the two tissues (Shade *et al.*, 2017). We also hypothesized that the majority of seed-associated microbes would be transient and that only a small fraction of the microbial taxa would be transmitted to the developing seedling (Hodgson *et al.*, 2014; Walitang *et al.*, 2019). We lacked a strong

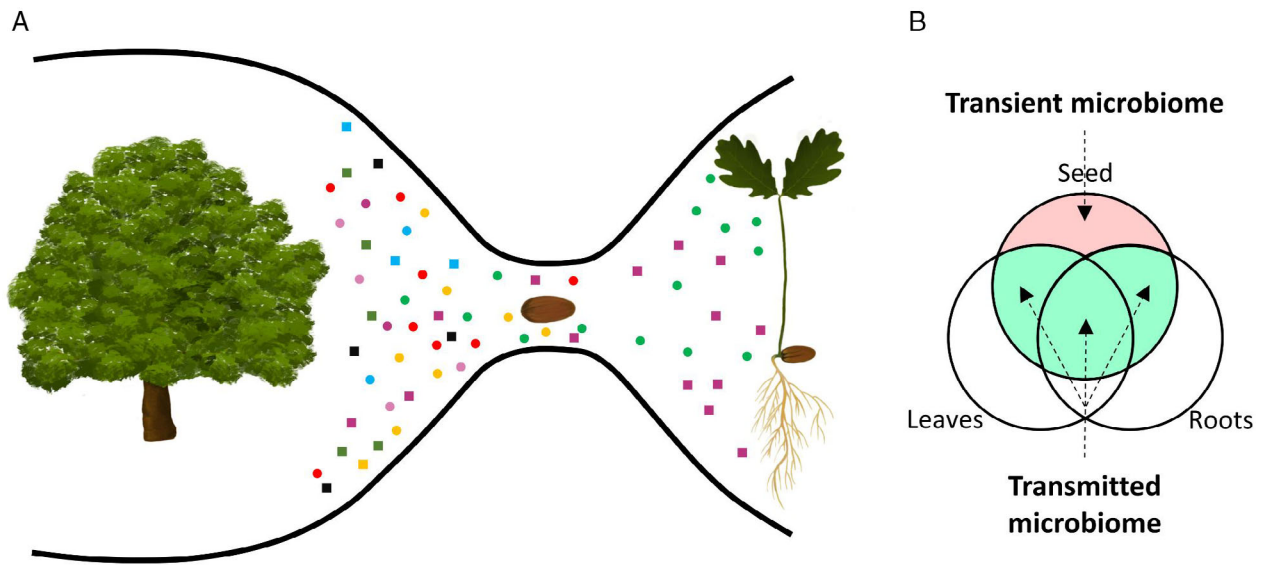


Fig. 1. Vertical inheritance and the transient and transmitted microbiome. Panel A shows the process of vertical transmission, with the hypothesized bottleneck at the seed level. Several studies have explored how microbes move from the parent tree into the seed (Rybakova *et al.*, 2017; Newcombe *et al.*, 2018), but we lack studies on the transmission from the seed to the seedling. Panel B shows a hypothetical Venn diagram illustrating the categorization of the seed microbiome into the transient (pink) and transmitted (green) microbiome.

prior on the subsequent distribution of the transmitted microbiome, but some studies have suggested that the composition of seed-associated microorganisms reflects the microbial community in the roots (Adam *et al.*, 2018; Kong *et al.*, 2019), while seed transmission to the phyllosphere is more limited (Dunleavy, 1989; Lopez-Velasco *et al.*, 2013).

Materials and methods

Microcosms

The culturing devices for growing plants in microbe-free condition were developed specifically for this work and are in the process of patenting at the Swedish patent and registration office (application number 1950987-6) and are henceforth referred to as microcosms (Fig. 2). The microcosms are made of autoclavable, transparent polymeric material which allows for photosynthesis and are designed to have three compartments: The upper and lower compartments are separated by a barrier (the middle compartment) to avoid cross-contamination between the plant's above- and belowground parts. The upper compartment, where the shoots and leaves grow, is equipped with a ventilation system with $\leq 0.22 \mu\text{m}$ filters, for plant respiration. The middle compartment consists of multiple layers of different materials to encapsulate the seed and separate the upper and lower compartment, and thereby stop the exchange of gas or liquid between the two compartments. The lower compartment, where the roots grow, contained only sterile Milli-Q[®] water.

Experimental design and sampling

Acorns of common oak (*Quercus robur* L.) were collected from a single oak tree located in Stockholm, Sweden (59°21'54.9"N 18°03'20.0"E) on October 20, 2018. The reason to use a single tree was to minimize the influence of oak genetic variation on the microbial community. Acorns were collected directly from the tree canopy, before falling onto the ground, to minimize the risk of microbial contamination from the environment, especially soil. The collected acorns were first rinsed in sterile Milli-Q water, then surface sterilized using sodium hypochlorite 10% for 30 min, followed by three rinses in sterile water, 5 min each. Surface sterilized acorns were stored in sterilized filter sand (0.4–0.8 mm) at 4°C until use. The used filter sand was first autoclaved twice at 121°C for 15 min with 24 h in between, then wetted with sterile Milli-Q to reach 30% soil moisture. At the start of this experiment, 48 acorns were re-surface sterilized in 5% sodium hypochlorite for 5 min, followed by three rinses in sterile water, 3 min each. Twenty-four of those were dissected using sterile razors, one for each acorn, separating the pericarp from the embryo (cotyledons, radicle and plumule) as two tissue types. The 24 embryo and 24 pericarp samples were then stored at –20°C until further processing. The other 24 acorns were planted individually in sealed 50 ml falcon tubes on wet, sterile cotton. Once germinated, acorns were aseptically transferred under a laminar flow hood to the microcosms containing sterile Milli-Q water without any addition of nutrients or minerals and placed in a growth chamber at 21°C with

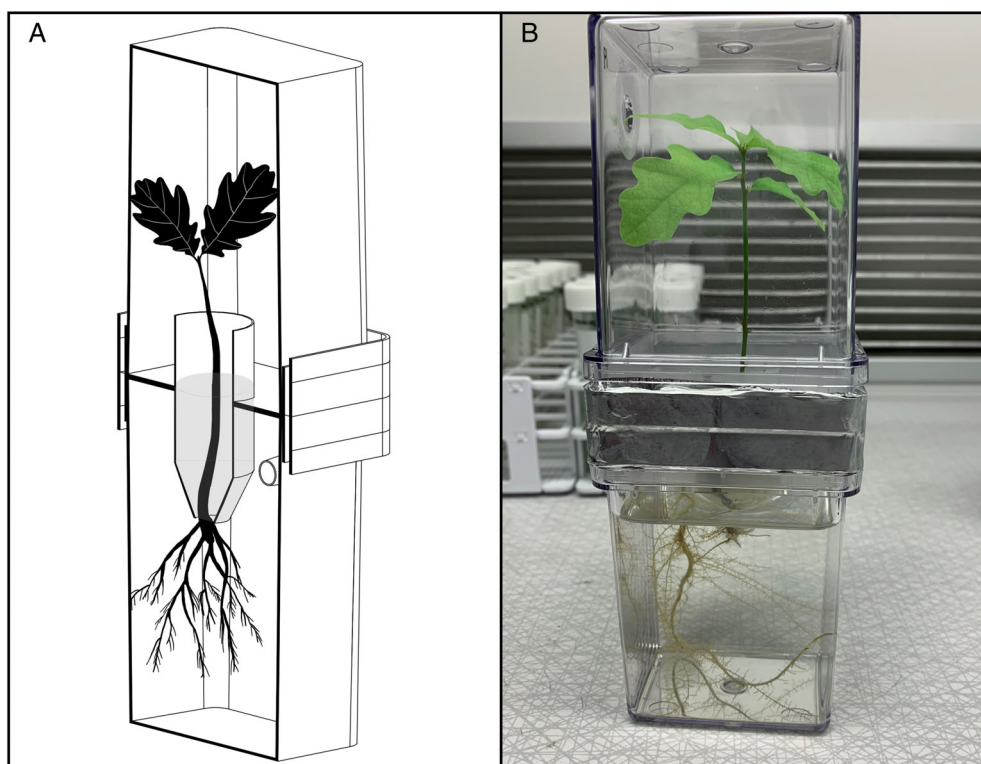


Fig. 2. Description of the culturing devices (microcosms) developed and used in this study. Panel A shows a schematic drawing of the microcosm design with an oak seedling. The middle compartment (grey area with the conical shape) consists of multiple layers of different materials to encapsulate the acorn and separate the upper and lower compartments, and thereby stop the exchange of gas or liquid between the two compartments. Panel B shows a photo taken of a seedling of the pedunculate oak *Quercus robur* growing inside a microcosm at the time of sampling. The lower compartment contained only sterile Milli-Q water without any additives.

16 h light for 6 weeks. For each seedling, aboveground (leaves and shoots or phyllosphere), and belowground (roots) plant parts were collected and stored at -20°C until lyophilization, making a total of 24 phyllosphere and 24 root samples. All samples ($n = 96$), were lyophilized in the ScanVac CoolSafe™ (LaboGene), ground using TissueLyser II (Qiagen), and had their DNA extracted using DNeasy PowerSoil Kit (Qiagen).

Libraries and sequencing

The fungal ITS2 region of the ribosomal DNA was amplified using ITS86R (Vancov and Keen, 2009) and ITS5 (White *et al.*, 1990) as described by Scibetta *et al.* (2018), and the prokaryotic 16S V4 region was amplified using 515F–806R (Caporaso *et al.*, 2011). All primers were modified to include Illumina adapters for multiplexing with Nextera XT index kit as described by the manufacturer. PCR reactions were conducted in a total volume of 25 μl containing 12.5 μl of KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA), 1.5 μl of each primer (10 μM), and 1 μl of DNA template. Reactions were incubated in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) for 3 min at 98°C

followed by 30 cycles of 30 s at 95°C , 30 s at 50°C and 30 s at 72°C . All reaction cycles ended with a final extension of 1 min at 72°C . Nuclease-free water (Qiagen, Valencia, CA, USA) replaced template DNA in negative controls. All amplicons, including amplification mixtures from negative controls, were sequenced using Illumina MiSeq V3 (2×300 bp) chemistry according to the manufacturer's instructions.

Data analysis

The quality of Illumina reads was checked using FastQC V 0.11.8 (Andrews, 2010). Trimmomatic V 0.39 (Bolger *et al.*, 2014) was used to clip Illumina adapters, filter read quality using a four-base wide sliding window, truncate reads when the average quality per base dropped below 15 and remove reads less than 100 bases long. Only paired reads, where both forward and reverse reads of the same sequences passed the quality filter, were assembled using PANDAseq V 2.1 with default parameters and read overlap of 20 bp (Masella *et al.*, 2012). Chimeric sequences were identified and removed using VSEARCH V 1.4.0 (Rognes *et al.*, 2016). Filtered reads were clustered using the UCLUST

algorithm (Edgar, 2010), as implemented in QIIME V 1.9.1 `pick_open_reference_otus.py` workflow (Caporaso *et al.*, 2010). The most abundant sequences in each operational taxonomic unit (OTU) were selected for taxonomic identification using BLAST (Altschul *et al.*, 1990). UNITE dynamic database V 8 released on 02.02.2019 (Abarenkov *et al.*, 2010) and Greengenes 13_8 (DeSantis *et al.*, 2006) were used for chimera identification, clustering and taxonomic assignment of fungi and bacteria respectively.

Diversity metrics and statistics

When calculating species richness and Shannon diversity, we accounted for uneven sequencing depth by first rarefying each sample to 300 reads. Diversity measures of different plant parts were then compared using non-parametric two-sample tests. MetagenomeSeq's cumulative sum scaling (Paulson *et al.*, 2013) was used as a normalization method to account for uneven sequencing depth for subsequent community composition analyses, including the calculation of Bray–Curtis dissimilarity metrics (Bray and Curtis, 1957), the construction of PCoA plots, and defining the core microbiome. The core microbiome, defined as OTUs present in at least 75% of the samples, was identified separately for embryos, pericarps, phyllosphere and roots. Pairwise comparisons between the community composition of different plant parts were done using *adonis* (~Permanova) from package ‘vegan’ (Oksanen *et al.*, 2007; Arbizu, 2019). The most prevalent taxa, which had a relative abundance >0.1% across all samples, were used to evaluate differences in relative abundance of the detected taxa between the different plant parts using Kruskal–Wallis method (Kruskal and Wallis, 1952). Significance in all analyses was determined using 999 Monte Carlo permutations, and Benjamini–Hochberg (FDR) corrections were used to adjust the calculated P-values. Cytoscape 3.7.2 (Shannon *et al.*, 2003) was used for network construction, and yFiles (Wiese *et al.*, 2002) was used to visualize the transient microbiome, transmitted microbiome, and unique and shared taxa between the different plant parts.

Results

Microbial community composition

After quality filtering and removal of chimeric and plant reads, amplicon sequencing yielded 4 843 432 ITS and 1 622 809 16S high-quality reads which were assigned to 4664 fungal and 19 172 bacterial OTUs. The ITS OTUs were assigned to five fungal phyla and 144 genera, and the 16S OTUs were assigned to 25 bacterial phyla and 216 genera. The fungal phyla *Ascomycota* 73%,

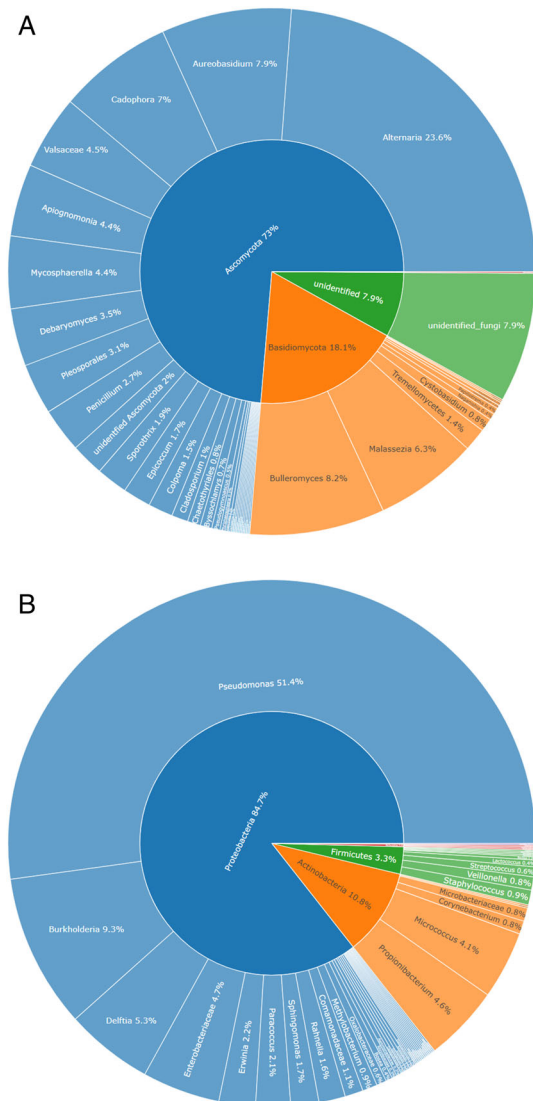


Fig. 3. Sunburst plot showing the most abundant fungal (A) and bacterial (B) phyla and genera and their relative abundance across the full set of samples taken from the embryo, pericarp, phyllosphere and root of the pedunculate oak *Quercus robur*.

Basidiomycota 18.1%, an unidentified phylum 7.9% and *Glomeromycota* 0.1% were the most abundant across all samples collectively (Fig. 3A). The fungal community was dominated by ascomycetous genera, *Alternaria* 23.6%, *Aureobasidium* 7.9%, *Cadophora* 7%, an unidentified genus belonging to the family Valsaceae (4.5%), *Apiognomonia* 4.4%, and *Mycosphaerella* 4.4%, and the basidiomycete genera *Bulleromyces* 8.2% and *Malassezia* 6.3% (Fig. 3A). Regarding the bacterial community, Proteobacteria 84.7%, Actinobacteria 10.8%, Firmicutes 3.3%, Bacteroidetes 0.6% and Saccharibacteria, formerly known as TM7 0.1% were the top phyla across all samples collectively (Fig. 3B). The most abundant bacterial genera within the phylum

Proteobacteria were *Pseudomonas* 51.4%, *Burkholderia* 9.3%, *Delftia* 5.3%, an unidentified *Enterobacteriaceae* 4.7%, *Erwinia* 2.2% and *Paracoccus* 2.1%, and the most abundant genera within the phylum *Actinobacteria* were *Propionibacterium* 4.6% and *Micrococcus* 4.1% (Fig. 3B).

Diversity and spatial distribution of the inherited microbiome

The microbial diversity and richness were twofold to fourfold higher in the embryo than in the pericarp (Fig. 4; Table S1). The fungal and bacterial community composition strongly differed between the embryo and pericarp (blue vs. red circles in Fig. 5; Table 1). The fungal community of the embryo was characterized by a dominance of the ascomycetous genera *Alternaria* 19.68%, *Debaryomyces* 10.39%, *Apiognomonia* 6.63%, unidentified *Valsaceae* 5.6%, unidentified *Ascomycota* 4.49% and *Mycosphaerella* 4.4%, the basidiomycete genus *Malassezia* 18.36% and unidentified fungi 9.57% (Fig. 6A). In contrast, the pericarp was 99.9% composed of ascomycetous genera, with the dominant genera being *Alternaria* 34.5%, *Aureobasidium* 17.9%, *Mycosphaerella* 11.47%, unidentified *Valsaceae* 11.26%, *Apiognomonia* 8.19% and *Epicoccum* 5.56% (Fig. 6C and Fig. S1). The bacterial community in the embryo was comprised of some 20 phyla, although only *Proteobacteria* 78%, *Actinobacteria* 13%, *Firmicutes* 7% and *Bacteroidetes* 1% had a relative abundance larger than 1%. The most abundant genera in the embryo were *Pseudomonas* 53%, *Propionibacterium* 8.6%, *Delftia* 6.1%, unidentified *Enterobacteriaceae* 4.3% and *Erwinia* 4.3%. Similar to fungi, the bacterial community of the pericarp was dominated by a single phylum (*Proteobacteria* 99%) (Fig. 6B). The high dominance of *Proteobacteria* in the pericarp was due to the presence of *Pseudomonas* 91.80% and *Erwinia* 4% (Fig. 6D).

Transient versus transmitted microbiome

The transient microbiome consisted of 36 fungal and 89 bacterial taxa (Fig. 7A and c). Among the transient microbiome, 27 fungal and 84 bacterial taxa were unique to the embryo, eight and five fungal and bacterial taxa were unique to the pericarp, and only one fungal and two bacterial taxa were shared between both. Interestingly, all taxa in the transient microbiome had a very low relative abundance and jointly accounted for less than 5% of the fungal and bacterial communities (Fig. 7B and D; Table S2). The transient community was dominated by the ascomycetous fungus *Pseudogymnoascus* 2.1% and the bacterial genus *Arcobacter* 0.9% belonging to the phylum *Proteobacteria*. The transmitted microbiome was characterized by a diverse community of 51 fungal and

70 bacterial taxa with high relative abundance, representing >95% of the phyllosphere and root fungal and bacterial communities in the developing seedling (Fig. 7; Table S2). Notably, unique taxa were identified in the phyllosphere and root microbial communities that were not found in seeds. These unique fungal and bacterial taxa included respectively, 39 and 41 taxa that were unique to the phyllosphere, 7 and 9 taxa that were unique to the roots, and 11 and 5 taxa that were shared between both tissue types. Similar to the transient community, all these taxa exhibited low abundance and cumulatively accounted for less than 5% of the total fungal and bacterial communities. A complete list of the transient, transmitted and unique taxa are presented in Table S2.

The transmission route from the seed to the belowground and aboveground tissues of the developing seedling

The embryo and phyllosphere had a very similar community composition and an equally high species diversity, while the pericarp and root each had a distinct microbial community with a much lower level of diversity (Figs 4 and 5). Although the fungal and bacterial taxa present in the acorn were present in the phyllosphere at a relative abundance similar to their relative abundance in the embryo, only a subset of the fungal and bacterial taxa present in the embryo and pericarp were found in the roots of the developing seedling (Figs 5, 6, and 7). Some genera exhibited a high relative abundance in roots but a low relative abundance in the embryo, pericarp and phyllosphere (e.g. *Bulleromyces*, *Cadophora* and *Burkholderia*; Fig. 6). This indicates strong differences between fungal and bacterial genera in their propensity to migrate to the roots, and/or that some genera proliferated more rapidly once they had migrated to the roots (Fig. 5; Table S3).

The core microbiome of the embryo consisted of five fungal and eight bacterial genera, the core microbiome of the pericarp consisted of nine fungal and three bacterial genera, the core microbiome of the phyllosphere consisted of eight fungal and five bacterial genera, and the core microbiome of roots consisted of six fungal and four bacterial genera (Table S4). Overall, 9 fungal and 10 bacterial genera were present in all four core microbiomes (Fig. 8). The relative abundance of part of the core microbiome greatly increased during vertical transmission and/or subsequent proliferation, resulting in a higher relative abundance of some fungal and bacterial genera in the phyllosphere or roots than in the embryo and pericarp. For example, *Micrococcus* (17.52%), *Paracoccus* (9.02%) and *Delftia* (16.60%) exhibited a high relative abundance in the phyllosphere but only represented a small fraction of the microbial

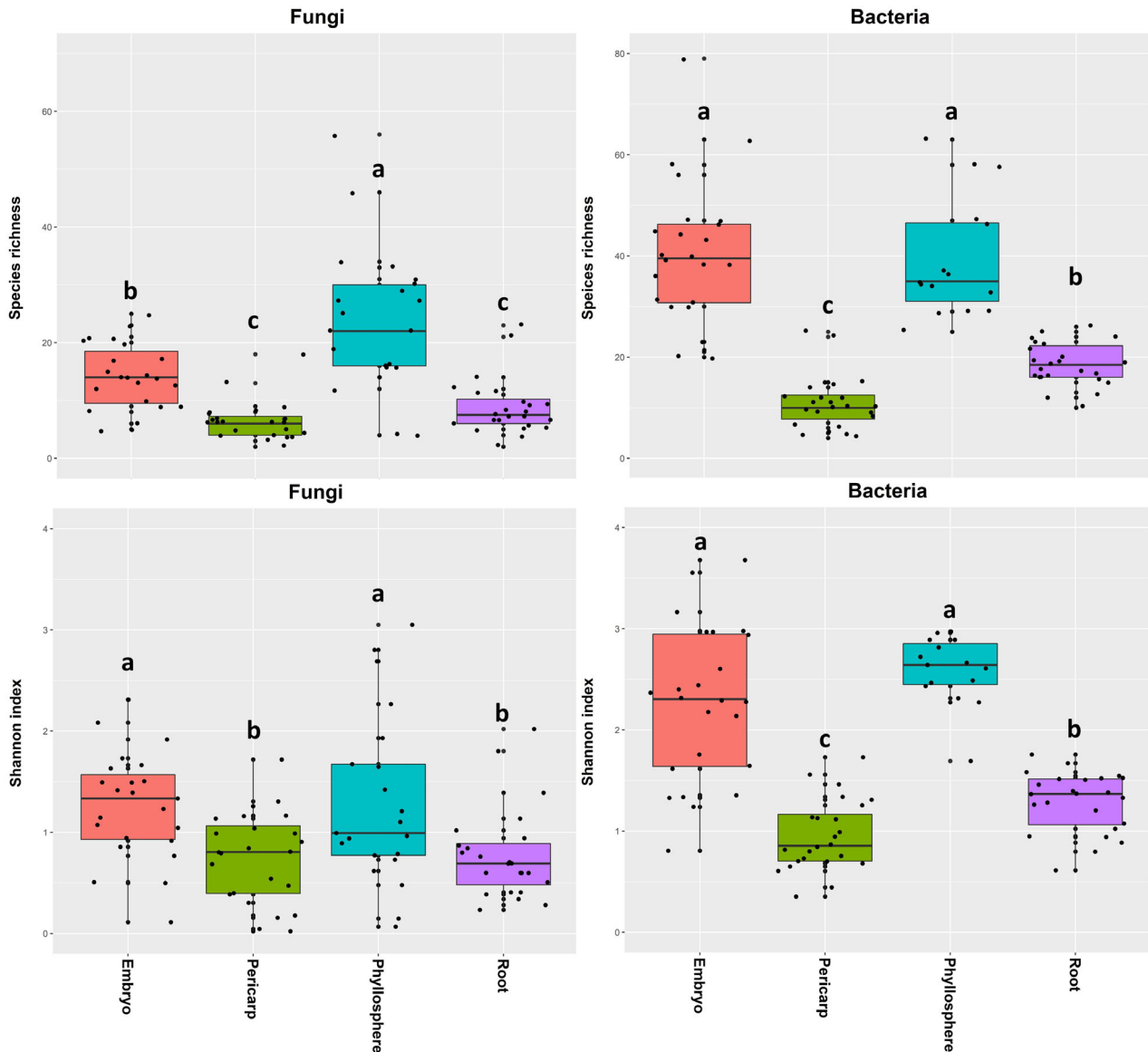


Fig. 4. Box plots showing the fungal and bacterial species richness and diversity (Shannon index) in the embryo, pericarp, phyllosphere and root of the pedunculate oak *Quercus robur*. Superimposed on the box plots are the horizontally jittered raw data points. Different letters indicate significant differences between groups ($P < 0.05$).

community present in the embryo and pericarp (Fig. 8; Table S3). Similarly, *Bulleromyces* (27.91%), *Burkholderia* (36.17%) and *Cadophora* (24.78%) comprised a large fraction of the root community but were at low relative abundance (<2%) in embryo and pericarp (Fig. 8; Table S3). In contrast, some taxa showed a low propensity to migrate or did not proliferate after migration. For example, *Erwinia*, *Colpoma*, *Epicoccum*, *Cladosporium* and unidentified *Chaetothyriales* were found to have a lower relative abundance in the phyllosphere and roots of developing seedlings than they did in embryo and pericarp.

Discussion

While the diversity of the plant microbiome, and its role for plant health, are increasingly recognized, we lack insights into the mechanisms behind the assembly of the plant microbiome and transmission of microorganisms from parent to offspring. In the current study, we investigated the transmission of the plant microbiome from seed to seedling, which is a crucial but largely overlooked part of the vertical inheritance process. We first dissected and sequenced individual seeds, which showed that the inherited microbiome is highly distinct within the seed

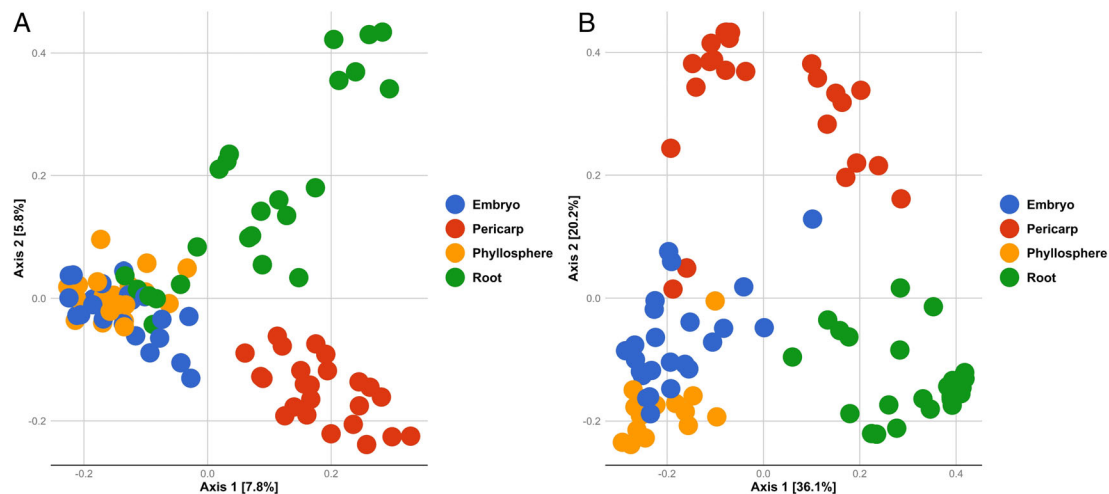


Fig. 5. PCoA based on Bray–Curtis dissimilarity metrics, showing the distance in the fungal (A) and bacterial (B) communities between embryos, pericarps, leaves and roots of the pedunculate oak *Quercus robur*.

Table 1. Pairwise comparisons between the fungal and bacterial communities in the embryo, pericarp, phyllosphere and root of the pedunculate oak *Quercus robur*.

	Embryo				Pericarp				Phyllosphere			
	Fungal		Bacterial		Fungal		Bacterial		Fungal		Bacterial	
	R^2	P-FDR	R^2	P-FDR	R^2	P-FDR	R^2	P-FDR	R^2	P-FDR	R^2	P-FDR
Pericarp	0.69	0.001	0.47	0.001	–	–	–	–	–	–	–	–
Phyllosphere	0.04	0.023	0.20	0.001	0.69	0.001	0.58	0.001	–	–	–	–
Root	0.19	0.001	0.62	0.001	0.49	0.001	0.48	0.001	0.18	0.001	0.67	0.001

The comparisons were based on Bray–Curtis dissimilarity and P -values were calculated using pairwise adonis and corrected using the FDR method.

(i.e. embryo vs. pericarp), with a particularly high microbial diversity within the embryo. We then used state-of-the-art culturing devices to grow common oak seedlings in the absence of environmental microbes, which showed that the major part of the inherited microbiome is transmitted from the seed to the seedling. While there was a strong imprint of the embryo's microbiome on the phyllosphere microbiome, the root microbiome represented a distinct subset of the seed microbiome. Collectively, the results provide clear evidence of microbial inheritance in plants, niche differentiation of the inherited microbiome in both seeds and seedlings, and divergent transmission routes from the acorn to the phyllosphere and roots. These findings shed new light on the source and transmission of plant-associated microbes and increase our understanding of the dispersal and distribution of microbes within natural environments.

The majority of previous studies on the seed microbiome used bulk samples (several pooled seeds) (Rybakova *et al.*, 2017; Adam *et al.*, 2018; Solanki *et al.*, 2019) which

inflates microbial diversity, especially when evaluating the transmission of the inherited microbiome into individual seeds (Gundel *et al.*, 2011; Newcombe *et al.*, 2018). The present study demonstrated that individual seeds possessed high microbial diversity, which was higher in the embryo than in the pericarp. These results are in contrast to other recent studies that reported a limited number of bacterial and/or fungal species present in seeds (Hodgson *et al.*, 2014; Newcombe *et al.*, 2018).

The composition of the microbial community in embryo and pericarp greatly differed. In particular, the bacterial community of the pericarp was almost completely dominated by the genus *Pseudomonas*. While this genus was also present in the embryo, it was part of a diverse community that included other bacterial genera. The existence of strong differentiation of the microbial community between different seed parts may be a general pattern, and in this case, the lower diversity and evenness in the pericarp may be due to the limited resources present in the pericarp. In this regard, it is interesting to note that no specialists or

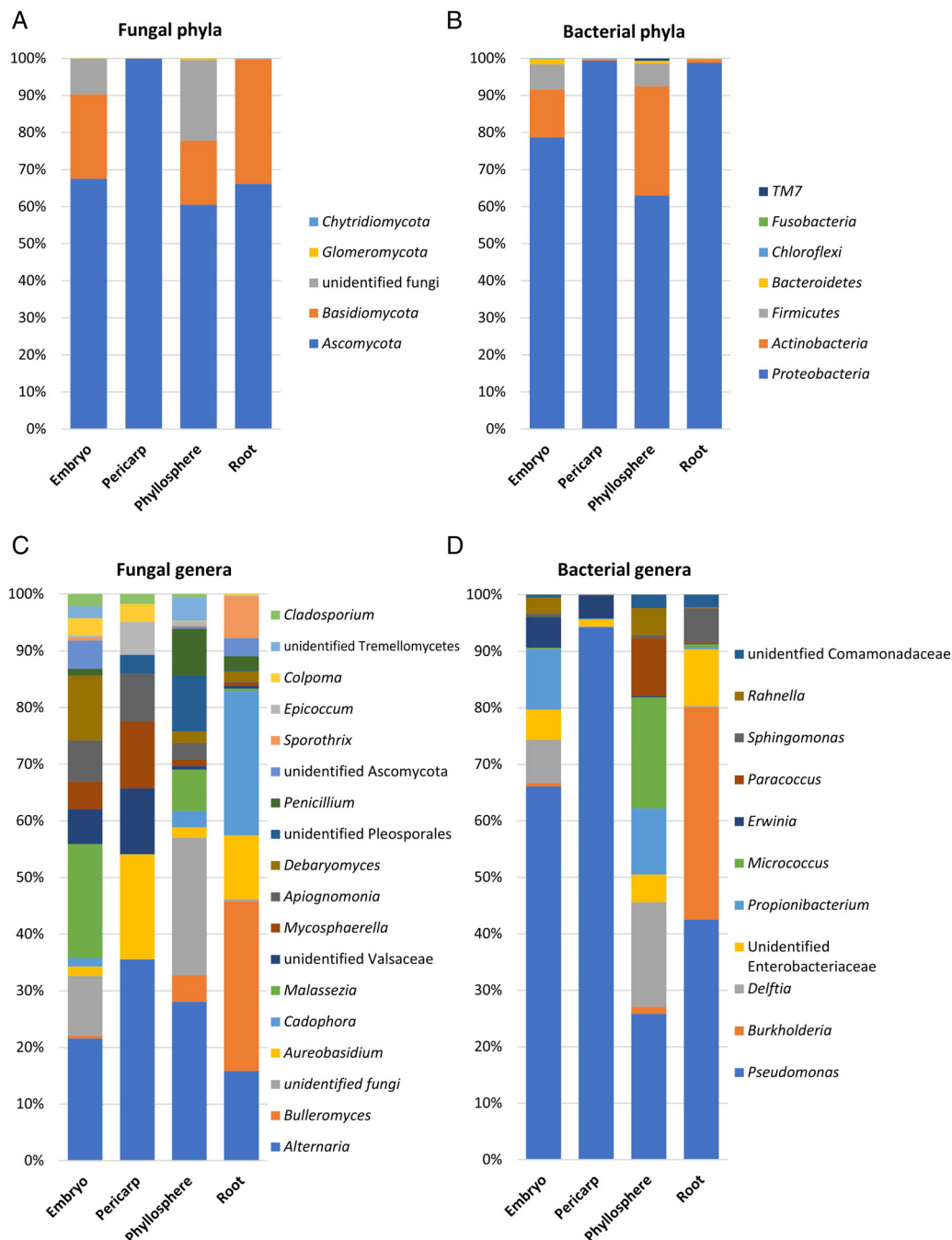


Fig. 6. Relative abundances of the most prevalent fungal and bacterial phyla (top) and genera (bottom) present in the embryo, pericarp, phyllosphere and roots of the pedunculate oak *Quercus robur*.

novel species were identified in the pericarp. Instead, OTUs belonging to a more generalist genus (*Pseudomonas*), known to thrive in many different habitat types (Weller, 1988; Weller, 2007), were identified in the pericarp. Although *Pseudomonas* species are generally considered plant and seed pathogens, they can also induce plant growth and secrete antibiotics into the rhizosphere (Burr *et al.*, 1978; Kumar and Dube, 1992; Raaijmakers and

Weller, 1998). Notably, despite their high abundance, seeds did not show any disease symptoms. While highly speculative, we suggest that *Pseudomonas* taxa may have a role in protecting the acorn from soil pathogens, and stimulate the development of the acorn, though direct evidence for this premise will still need to be provided. In contrast to the pericarp, several unidentified fungal and bacterial taxa were identified in the embryo, highlighting the scarcity of studies

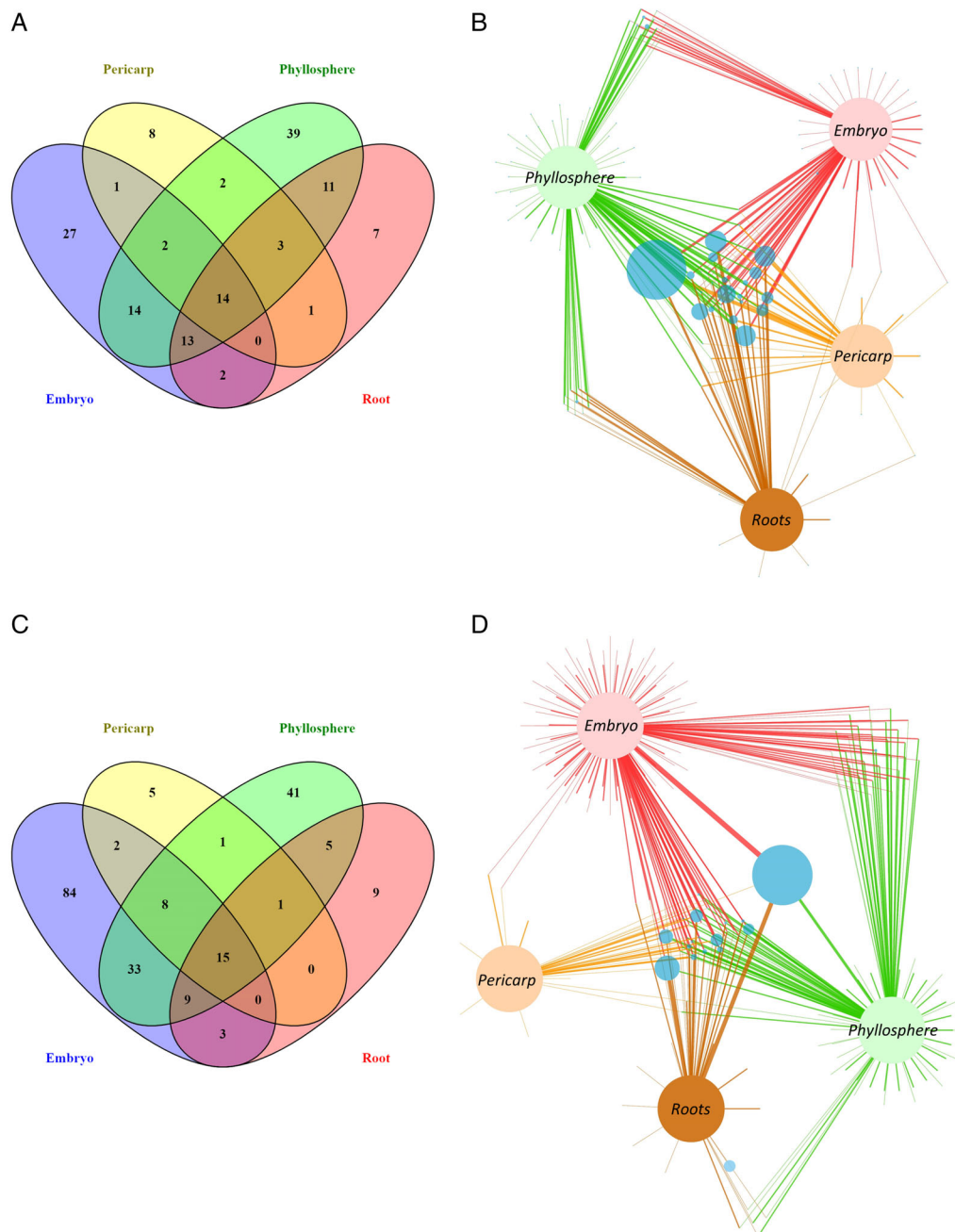


Fig. 7. Venn diagram and network graphs showing shared and unique fungal (A and B) and bacterial (C and D) genera in the embryo, pericarp, phyllosphere and root of the pedunculate oak *Quercus robur*. Panels B and D are networks created using Cytoscape 3.8.0, with edges and nodes organized using yFiles Organic layout. Blue nodes represent microbial genera, and pink, beige, brown and green represent embryo, pericarp, roots and phyllosphere respectively. Edges are coloured according to the plant tissue they belong to and edge widths are weighted to reflect the relative abundance of the genera. The figure shows that the most abundant genera were shared between the acorn and seedling's phyllosphere and roots.

on seed microbes and the potential for the discovery of novel species that occupy this niche. Although not documented in the present study, it is expected that the inherited microbiome in the acorn primarily originated from the microbiome of the parent tree. A recent study of the maternal effect on the fungal composition of seeds indicated that the

composition of the tree microbiome had a major influence on the microbial seed community (Fort *et al.*, 2019). Other sources of the seed microbiome include floral pathways, via the stigma of flowers, and direct contamination of seeds by microorganisms present in the environment (Maude, 1996; Shahzad *et al.*, 2018).

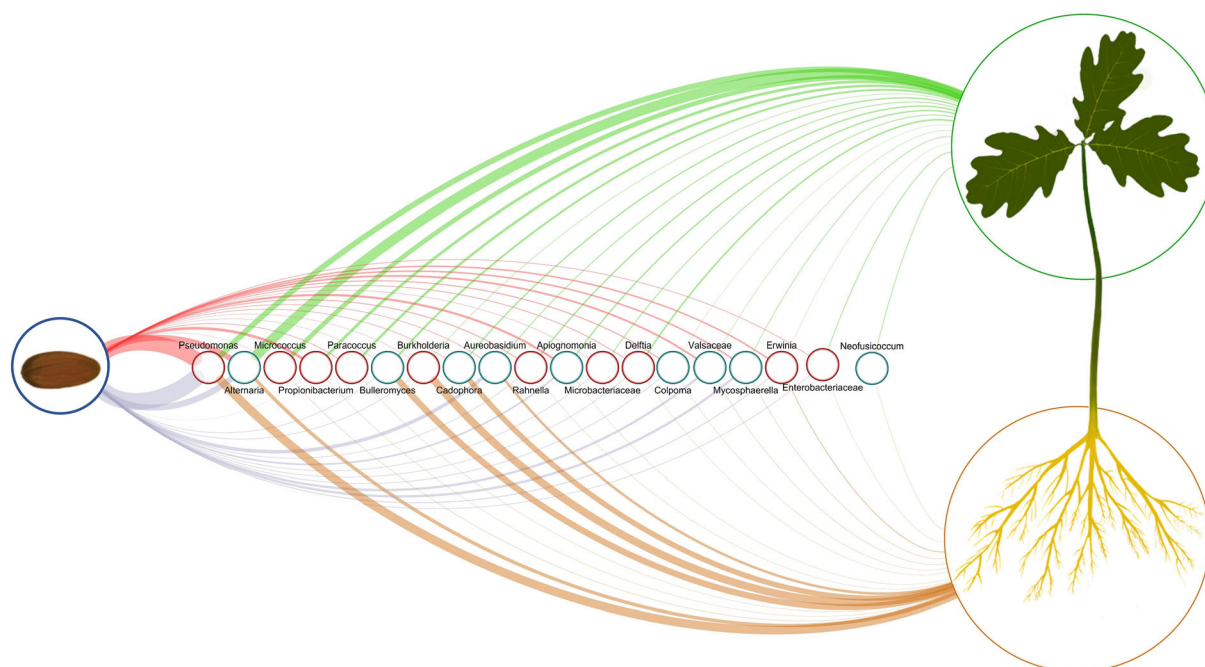


Fig. 8. Core microbiome distribution and niche differentiation of the vertically transmitted microbiome of the pedunculate oak *Quercus robur*. Line colours correspond to sample types (pink: embryo, purple: pericarp, brown: roots, green: phyllosphere). The width of the line between each genus and sample type reflects the relative abundance of the genus in that particular sample type. Blue circles represent fungi, red circles represent bacteria.

Contrary to our prediction, the majority of fungal and bacterial taxa (>95%) of the inherited microbiome were present in the transmitted microbiome in the phyllosphere and roots of germinated seedlings. Although vertical transmission of the microbiome represents a strategy that allows a host to provide their offspring with mutualistic endosymbionts (Ferreira *et al.*, 2008), some of the transmitted microorganisms may have detrimental effects, as has been reported for the plant and insect microbiome (Neergaard, 1977; Ewald, 1987; Wong *et al.*, 2013; Douglas, 2015). Nevertheless, the fact that the transmitted microbiome represented a very large fraction of the seed microbiome further emphasizes the ecological role of seeds as a reservoir and source for community assembly in new seedlings (Shade *et al.*, 2017). In contrast to the transmitted microbiome, the transient microbial community consisted of several taxa with very low abundance, including the genera *Pseudogymnoascus*, *Arcobacter*, *Cystofilobasidium* and *Rutstroemia*. While it has been argued that the seed represents an end-point for transient microorganisms, the seed may also provide an effective strategy for the dispersal of transient microbes into new environments, or transient microbes may play a role in the decomposition of (parts of) the acorn after dispersal.

Although the fungal and bacterial community in the phyllosphere reflected the community present in the embryo, only part of the microbes present in the embryo

and pericarp were identified in the roots of the developing seedling. As a result, the microbial community of the phyllosphere differed significantly from the root community, despite originating from the same source. These differences could be due to a combination of microbial life-history traits and plant regulatory factors that limit the migration of some members of the seed microbiome into the roots. Whether direct or indirect, plant-mediated or microbe-mediated, results of the present study indicate that the distinct microbial communities commonly reported between above- and belowground plant parts in natural and agricultural studies may originate during the transmission of microbes from seed to seedling, and not, as commonly assumed, due to inherent differences in the microbial communities in the soil and air. Such initial seed-borne differences among the below- and aboveground plant tissues may cause strong priority effects during later development. Our demonstration of partial transmission of the seed microbiome to the roots confirms previous work suggesting that plant seeds are a repository for rhizosphere microbial communities (Kong *et al.*, 2019), and the high resemblance of the embryonic and phyllosphere microbiome indicates that seeds may play an even larger role as a repository for the phyllosphere microbial community.

We do note that the microbial communities we found in the offspring phyllosphere and especially the roots are

expected to differ from those found in natural systems. First, acorns used in our study were collected directly from the canopy before falling onto the ground. In this regard, the fungal community of acorns has been reported to undergo a shift after falling to the ground and have a greater resemblance to the soil community (Fort *et al.*, 2019). The overwintering period is also expected to allow several microbial species to penetrate through the pericarp and establish themselves in both pericarp and embryo. Importantly, the oak seedlings in the present study were grown in sterile chambers and in sterile ultrapure water, the latter imposing anaerobic condition on the plant roots that may have favoured the growth of some anaerobic taxa and limited the growth of other taxa. The fact that we did not add any additional nutrients to the water may also have inhibited the growth of some nutrient-dependent microorganisms (Bing *et al.*, 2018; Moccia *et al.*, 2020). On the other hand, this approach allowed us to eliminate those factors that might have altered the microbial community transmitted to the developing seedling. Despite the expected difference, it is likely that the microbiome that is transmitted from the acorn to the roots would still play a role in plant growth and survival, before being altered or even outcompeted by the soil microbiome (Mitter *et al.*, 2017).

Some taxa, albeit at a relatively low abundance, were detected in the phyllosphere and roots of the developing seedling but not in seed (embryo and pericarp) tissues. Although it is difficult to pinpoint the exact reason behind this, there are several non-mutually exclusive explanations. A biological-statistical explanation is that there is a natural variation between individual acorns. Thus, there is a statistical expectation of slight differences in taxa present in acorns used to characterize the microbial community of the seed versus the acorns used to germinate seedlings. This would naturally result in some microbes not being present in the set of sequenced embryos and pericarps. A methodological explanation is also plausible in which some taxa that were initially below detection levels proliferated after their transmission to the developing seedling. A third explanation is that the experiment had low levels of contamination. Regardless of the reason(s) underlying the presence of unique taxa in the developing seedling, we chose to focus on the core microbiome in an attempt to identify those taxa that have a consistent presence in the acorn, and that were consistently transmitted to the developing seedling. Such core taxa are expected to play a significant functional role in the respective compartments of the holobiont (Vandenkoornhuys *et al.*, 2015).

In this regard, several taxa were found to be vertically transmitted include taxa with various functions such as the production of antimicrobial compounds, detoxification, nutrient uptake and growth-promoting activities. For

example, *Burkholderia*, the most abundant genus in the core microbiome of roots, have been reported to have plant-growth-promoting, nitrogen-fixing, and plant protection properties (Van Dommelen and Vanderleyden, 2007; Elliott *et al.*, 2009; Huang *et al.*, 2017; Zhang *et al.*, 2017). In addition to their interactions with plants, some members of *Burkholderia* have been shown to have an impact on fungal reproduction and were suggested to be part of the mycorrhizal intracellular microbiome (Nguyen and Bruns, 2015; Hassani *et al.*, 2018). The genus *Cadophora* comprises several root endophytes with antimicrobial activity (Tan *et al.*, 2018; Egidi *et al.*, 2019). *Cadophora* species were also found to produce high levels of plant cell wall-degrading enzymes, as well as small, secreted proteins and aquaporins that typically function in tissue colonization and nutrient acquisition in the intercellular spaces of host plants (Lo Presti *et al.*, 2015; Knapp *et al.*, 2018).

The core phyllosphere microbiome included bacteria such as *Micrococcus*, *Propionibacterium*, *Paracoccus* and *Delftia*. *Micrococcus*, a genus with widespread occurrence in a variety of different environments, have been previously isolated as endophyte from coffee seeds and banana leaves, and several species of *Micrococcus* are involved in the detoxification or biodegradation of the toxic carbamate pesticide, carbaryl and several other environmental pollutants (Doddamani and Ninnekar, 2001; Zhuang *et al.*, 2003; Vega *et al.*, 2005; Thomas and Soly, 2009). *Propionibacterium*, a bacterial endophyte previously reported to inhabit apple stems, has been suggested to play a role in solubilizing phosphate into phosphorus via the synthesis of propionic acid (Liu *et al.*, 2018). *Paracoccus* is a genus with high biochemical value due to its ability to degrade a wide range of compounds, including denitrification and sulfonate degradation. *Paracoccus* is also the only known bacterium that possesses all the components of the mitochondrial respiratory chain and oxidative phosphorylation pathway (John and Whatley, 1975). Species in this genus were reported to be a common endophyte in plant seeds (López-López *et al.*, 2010; Huang *et al.*, 2017; Liu *et al.*, 2017). *Delftia* species are seed bacterial endophytes known for their antagonistic activity against several plant pathogens, growth-promotion, and nitrogen-fixing properties (Han *et al.*, 2005; da Silveira *et al.*, 2019; Chen *et al.*, 2020).

Conclusion

In the present study, an attempt was made to overcome the challenges that exist in distinguishing between microbial communities that transmit vertically from the seed to a developing seedling from those that are acquired horizontally from the environment, and the ability to discern the vertical transmission routes from the seed to the

seedling. A novel, state-of-the-art culturing device was constructed and used to grow seedlings in a microbe-free environment while keeping belowground and aboveground tissues separated. The conducted study revealed that the inherited microbiome exhibited a high fungal and bacterial diversity and that a large part of the seed microbiome is transmitted to the phyllosphere and roots of the developing seedling. The collective data provide clear evidence of vertical transmission from seed to seedling and highlight the important role of vertical transmission during the assembly of the plant microbiome. A promising avenue for future research is to study the continuity of this transmitted microbiome across generations, for example by monitoring the seed, plant and environmental microbiome during multiple plant generations. From an ecological perspective, new insights are provided on the distribution, dispersal and persistence of plant-associated microbes in natural and agricultural settings. The study also raises questions regarding how microbial assemblages are selected for during evolution in natural systems and the breeding of crops. Knowledge of the mechanisms of microbial inheritance in plants and a greater understanding of their functional role in plant fitness can lead to the development of strategies for breeding crops with greater disease resistance and adapted to stressful environments.

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Authors' Contributions

AA and AJMT conceptualized and designed the experiment. AA performed the experiment and analysed the data. AA wrote the first draft, and AJMT made a major contribution to the final version. MW and LS performed the sequencing and contributed to the interpretation of the results and writing of the manuscript.

Data Availability Material

The datasets generated during the current study were deposited and are available at the National Centre for Biotechnology Information (NCBI), Sequence Read Archive (SRA), under the accession number PRJNA627957 (<http://www.ncbi.nlm.nih.gov/bioproject/627957>). Other data generated or analysed during this

study are included in this published article and its additional files.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Fungal and bacterial distribution and niche differentiation of the inherited microbiome within acorns of the pedunculate oak *Quercus robur*. Line colour corresponds to sample type (pink: embryo, orange: pericarp), circle size corresponds to the overall abundance of a taxon, and line width reflects the relative abundance of a taxon in that particular sample. Circle colours indicate phylum affiliation (see legend).

Table S1. Pairwise comparisons between the fungal and bacterial species richness and Shannon diversity in the embryo, pericarp, leaf and root of the pedunculate oak *Quercus robur*. The comparisons were based on rarefied OTU tables and P-values were calculated using pairwise t-tests and corrected using FDR method.

Table S2. List of transient and transmitted fungal and bacterial taxa, their relative abundance and taxonomic identity.

Table S3. List of fungal and bacterial taxa with different relative abundance in the embryo, pericarp, leaf, and root of the pedunculate oak *Quercus robur*. The comparisons were based on rarefied OTU tables and P-values were calculated using Kruskal–Wallis test and corrected using FDR method.

Table S4. List of core fungal and bacterial OTUs of the embryo, pericarp, leaf, and root of the pedunculate oak *Quercus robur* and their corresponding taxonomy.