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The effects of tree diversity and neighborhood on phyllosphere fungal communities

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ABSTRACT

Phyllosphere fungi, encompassing both epiphytic and endophytic fungi, have rarely been studied along gradients of host diversity. In this study, we used a tree biodiversity experiment to investigate how tree species richness, host and neighboring species identities, and host-mycorrhizal association affect the richness and composition of the whole phyllosphere community as well as that considering epiphytes and endophytes separately. To distinguish epiphytes from endophytes, we employed a leaf-surface sterilization treatment. Richness and composition of the mycobiome were quantified using next-generation amplicon sequencing. Our findings revealed that tree species richness affected only the overall phyllosphere fungal community, not endophytes alone, suggesting that neighborhood effects predominantly influence fungi on the leaf cuticle. Neighborhood effects were only detectable in the phyllosphere as a whole and not within endophyte and epiphyte communities. Fungal community composition was shaped by host species identity, tree richness, host mycorrhizal type and the combination of mycorrhizal types at the plot level. This study underlines the importance of analyzing epiphytes and endophytes separately and highlights the necessity of using leaf-surface sterilization when examining phyllosphere fungal communities.

1. Introduction

Leaves provide a highly versatile habitat for microorganisms, collectively referred to as the phyllosphere (Kembel and Mueller, 2014). Host species identity is a key determinant of microbial phyllosphere community composition (Higgins et al., 2014; Kembel and Mueller, 2014). In addition, plant diversity has been shown to influence phyllosphere communities, for example, pathogenic fungal infestations decrease with increasing host diversity, whereas pathogen richness increases (Hantsch et al., 2014a). These patterns are largely driven by dilution effects, where a higher proportion of diverse neighboring trees reduces the likelihood of encounters with specific pathogens (Keesing et al., 2006; Fig. 2A). Additionally, heterospecific neighbors can act as transmission barriers for host species-specific fungi, thereby reducing transmission rates at lower host densities (Keesing et al., 2006). Conversely, heterospecific neighborhoods may promote spillover effects if neighboring species are closely related or serve as competent alternative hosts, leading to increased pathogen transmission rates between hosts (Keesing et al., 2006; Rutten et al., 2021; Fig. 2B). In addition to local host diversity, the identity of local neighbor tree species strongly influences phyllosphere fungal communities. For example, the presence or absence of certain tree species in the vicinity can positively or negatively affect fungal pathogen infestations (Hantsch et al., 2013). Thus, both higher diversity in the local neighborhood and the specific composition of neighbor tree species can reduce fungal transmission through dilution effects or increase it via spillover effects (Kambach et al., 2021; Caballol et al., 2022; Fig. 2).

Phyllosphere fungi are broadly classified into two subgroups based on their location within the leaf. Epiphytic fungi, or epiphyllous fungi, grow on the leaf surface, including on the cuticula and epicuticular waxes, and comprise lichenized fungi, free-living yeasts and hyphomycetes (Fonseca and Inácio, 2006; Pinokiyo, A. 2006; Gomes et al., 2018).

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Many plant pathogens are classified as epiphytic, causing harm by penetrating leaf cells, extracting nutrients, and reducing transpiration and photosynthesis rates, thereby decreasing overall plant performance and fitness (Hajji et al., 2009; Yamazaki et al., 2008).

In contrast, endophytic fungi inhabit the interior of leaves (Arnold, 2007; Gomes et al., 2018). In the strict sense, endophytes are considered to be asymptomatic, causing no visible necrosis or structural modification of host tissues (Petrini et al., 1991). Endophytic fungi are ubiquitous in leaves and form a key component of the plant microbiome (Arnold, 2007; Massimo et al., 2015). Some are mutualistic promoting plant growth (e.g. *Epichloë*) (König et al., 2018), while others are latent pathogens (e.g. *Alternaria*) (Bastías et al., 2021; DeMers, 2022). Mutualistic endophytes can enhance the host's resistance to abiotic stress (e.g. drought or heavy metals) or to pathogens by parasitizing pathogenic fungi or inducing chemical defenses (Rodriguez et al., 2008; Latz et al., 2018; Araújo et al., 2020; Rajani et al., 2021). However, under high-stress conditions, many endophytes, including *Alternaria*, *Fusarium* or *Epichloë* species, can shift to a pathogenic state (Álvarez-Loayza et al., 2011; Constantin et al., 2020). Additionally, a fungal species occurring as an endophyte in one host can act as a pathogen in a neighboring host (Newcombe et al., 2020).

Studies on phyllosphere fungi have largely focused on pathogenic fungi with visible symptoms, while effects of host diversity on asymptomatic endophytes remain underexplored (Hantsch et al., 2014b; Rutten et al., 2021; Saadani et al., 2021). A decrease of fungal endophyte richness and diversity with increasing tree species richness was found in the BiodiversiTREE diversity experiment in Maryland (Griffin et al., 2019). Already within the first three years after establishing the experiment, functional guild richness and diversity, including pathogens, saprotrophs, and parasites, decreased with increasing tree species richness. In the subtropical BEF-China forest experiment, the richness of phyllosphere fungi was found to be more closely related to proportion of a particular host tree species in the local neighborhood than to overall tree species richness (Saadani et al., 2021), which clearly points to dilution effects (Civitello et al., 2015).

To date, studies on the phyllosphere microbiome have employed either culture-based approaches (Pocock and Duckett, 1984; Mostert et al., 2000) or high-throughpout sequencing (Purahong et al., 2019; Kambach et al., 2021), each with limitations. Culture-based methods fail to capture the majority of endophytic fungi, as most are unculturable (Wu et al., 2019), while high-throughput sequencing may detect non-viable fungal spores or DNA from killed pathogens (Hart et al., 2015; Masumoto and Degawa, 2019). Combining both methods revealed a low overlap in the taxa identified (Nelson and Shaw 2019). Studies have demonstrated that most (>90 %) endophytic taxa belong to the Ascomycota, with about 75 % of reported endophytic taxa falling into the classes Dothideomycetes and Sordariomycetes, with smaller contributions by Pezizomycetes and Leotiomycetes (Arnold, 2007; Unterseher et al., 2013; Higgins et al., 2014). Additionally, recent high-throughput sequencing studies identified many Basidiomycetes, e.g. Exobasidiaceae and Filobasidiaceae, as common endophytes (Fonseca and Inácio, 2006). Phyla like Chytridiomycota, Mucoromycota and Mortierellomycota are commonly found in low abundances (Peršoh, 2013; Kambach et al., 2021).

A way to differentiate epiphytic and endophytic fungi is the use of leaf-surface sterilization (Santamaría and Bayman 2005; Yang et al., 2023). Eliminating surface microbes through chemical and physical treatment (Masumoto and Degawa, 2019; Guzmán et al., 2020). However, only a limited number of studies have utilized surface sterilized and non-sterilized samples for comparative analyses, particularly in the context of tree diversity research (Gomes et al., 2018; Yang et al., 2023). Since epiphytic fungi generally exhibit higher richness and abundance than endophytes, environmental impacts on fungal communities may be misinterpreted if these groups are not analyzed separately (Santamaría and Bayman 2005; Gomes et al., 2018).

Interactions between microbial communities in different plant compartments must also be considered, as these compartments function as interconnected habitats within the host plant, and the host itself can be considered a holobiont (Cregger et al., 2018). The microbial communities of different compartments interact with each other directly via chemical signaling and vertical transmission or indirectly by influencing the host's physiology and nutritional status (Smith and Read, 2008; Hassani et al., 2018; Tedersoo and Bahram, 2019). Mycorrhizal fungi are able to affect the phyllosphere communities (Novas et al., 2009; Purahong et al., 2019; Debray et al., 2022), including both ectomycorrhiza (EM), formed mainly by interactions with Basidiomycota and less commonly with Ascomycota, and arbuscular mycorrhiza (AM), composed exclusively of Glomeromycota (Tedersoo et al., 2014). It was shown that mixing plants of these two mycorrhizal types in an experimental setup resulted in an spillover of EM fungi to AM plants and vice versa (Heklau et al., 2021). While past work has shown how mycorrhizal and phyllosphere fungi interact when infecting a plant (e.g. Liu et al., 2011; Debray et al., 2022), it is not known whether and how the host plant's mycorrhizal type affects phyllosphere fungal communities.

This study uses the MyDiv experiment, which manipulates tree species richness and plot mycorrhizal type, to investigate how fungal communities in the whole phyllosphere (non-sterilized leaf surface), the endophytic compartment (sterilized leaf surface) and the epiphytic compartment (phyllosphere excluding endophytes) are influenced by tree diversity and tree mycorrhizal type. We hypothesized (H1) that the richness and composition of fungal taxa differs between the phyllosphere, endophytic and epiphytic communities. We further hypothesized (H2a) that fungal richness and community composition are influenced by host identity and neighborhood plant diversity, with (H2b) potential dilution or spillover effects from neighbor plant species. Finally, we hypothesized (H3) that fungal community composition is affected by (H3a) the host's mycorrhizal status (AM or EM) or (H3b) the mycorrhizal type composition of the tree community (exclusively AM or EM hosts or, or a mix of both host types).

2. Materials & methods

2.1. Experimental design

Leaves were collected from the MyDiv experiment located at the Bad Lauchstädt Experimental Research Station of the Helmholtz Centre for Environmental Research - UFZ in Central Germany (Fig. 1A). The site is situated at 114–116 m a.s.l., experiences a continental summer-dry climate with an annual mean precipitation of 484 mm and annual mean temperature of 8.8 °C (Ferlian et al., 2018). Prior to 2012, the site was in agricultural use, afterwards converted to grassland for two years, and subsequently plowed in preparation for planting.

In March 2015, 80 plots (11 × 11 m) were established, containing 140 tree individuals each. Two-to three-year old trees were planted at a distance of 1 m. The experiment includes ten tree species, of which five are predominantly associated with either AM or EM. These tree species were planted in a gradient of species richness: monocultures, two-species, and four-species mixtures. The two-species and four-species mixtures consist of either only AM trees, only EM trees, or a combination of AM and EM trees. Consequently, each plot is characterized by either monotypic (AM or EM) or mixed (AM and EM) mycorrhizal status (Ferlian et al., 2018).

For this study, four species from each mycorrhizal type were selected: *Acer pseudoplatanus* L., *Fraxinus excelsior* L., *Prunus avium* L. (L.) and *Sorbus aucuparia* L. for AM trees, and *Betula pendula* Roth, *Carpinus betulus* L., *Fagus sylvatica* L. and *Quercus petraea* (Matt.) Liebl. for EM trees. Two species were excluded from sampling, the AM species *Aesculus hippocastanum* L. and the EM species *Tilia platyphyllos* Scop. This resulted in 672 samples from 42 plots, representing 32 species combinations (Appendix Table S1).

Sampling took place from 17 August to 10 September 2021. Samples were taken from a set of four adjacent trees, in the following called tree

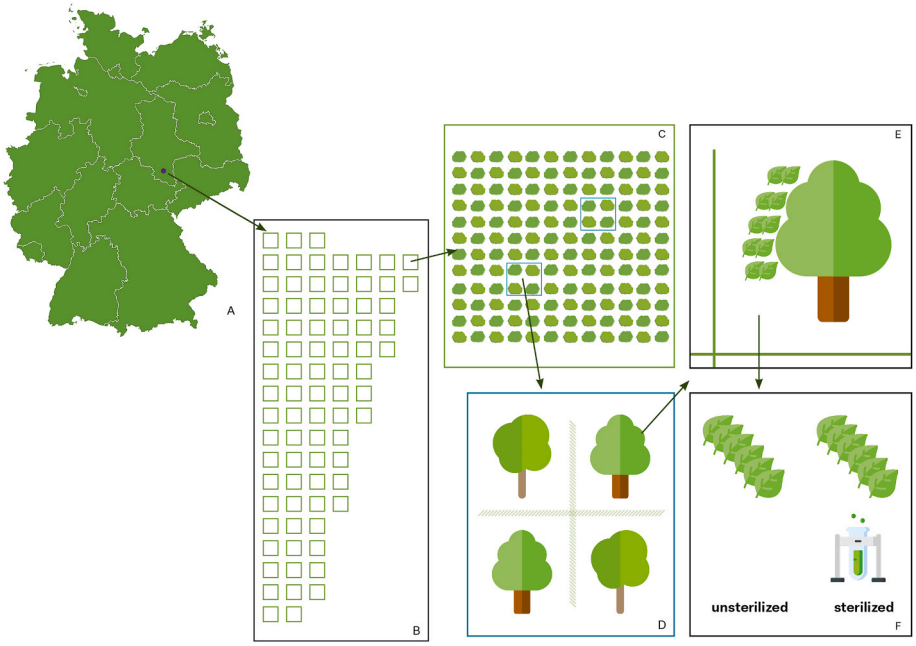

Fig. 1. Study design within the MyDiv Experiment, showing the location of the experiment in Germany (A) and schematic overview of the experiment (B). In each plot (C) two tree species quartets were chosen (D), marked in blue squares within the core area of 8 x 8 trees. In the center of the four individual trees is an interaction zone of the four individual trees, marked by two green lines symbolizing the interaction planes (D,E). Along this central interaction point we sampled two leaves at five heights, distributed equally over the tree (D). One leaf of each height was collected for the non-sterilized and sterilized treatment resulting in total of five leaves per treatment (F). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

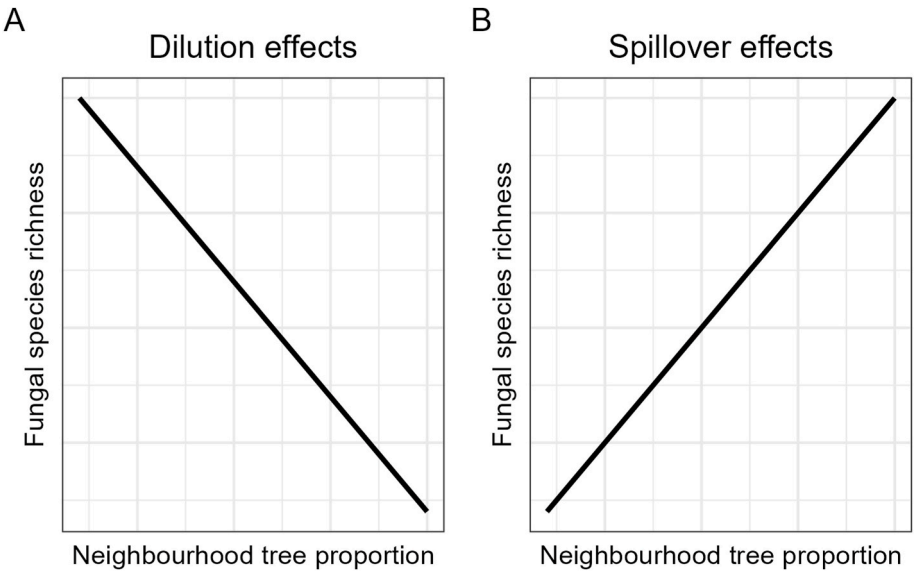

Fig. 2. Schematic overview of expected neighborhood effects on fungal species richness. A) Dilution effects are present if fungal species richness in a target host species is found to decrease with increasing frequency of a different non-target tree species in the local neighborhood. B) Spillover effects occur when fungal richness increases in a target tree species with increasing frequency of a different non-target tree species in the local neighborhood.

species quartets (TSQ) (Fig. 1C, D). For details on the TSQ design, see Trogisch et al. (2021) and Castro Sánchez-Bermejo et al., 2024. The four individual trees of one TSQ belonged to either one, two, or four different tree species. The design was chosen to capture the interactions of all tree species present in a single plot. We established two TSQs per plot, and - if possible - TSQs were chosen within the core area (8 m × 8 m). Sampling was conducted in the interaction zone of the four TSQ partners. The interaction zone is defined as the part of the canopy where the individual TSQ partners potentially have the highest level of interaction. Consequently, there is always an interaction zone between two neighboring trees, with a further interaction zone in the center of the TSQ where all four TSQ partners are potentially interacting. This mutual interaction was the focus of our sampling (represented by the lines in Fig. 1D, E). Leaves were then sampled across a height gradient within the interaction zone (Fig. 1E). From each tree, we collected two leaves from five different heights across the height gradient of the tree to capture some of the variation in the fungal community composition of individual leaves across the tree. This resulted in a total sample size of five leaves each per tree for leaf-surface sterilization and for the control, which were then pooled into two samples per tree (Fig. 1F). We conducted leaf-surface

sterilization to investigate the endophytic community, while the non-sterilized control samples depicted the full phyllosphere community containing both epi- and endophytic taxa. All sampled leaves were immediately stored at 4–8 °C in the field and further processed within 8 h.

2.2. Leaf-surface sterilization

Leaf-surface sterilization was conducted in 500 ml glass beakers following the protocol of Guerreiro et al. (2018). Each sample, consisting of five leaves, was processed as a single unit. Leaves were thoroughly washed in ddH2O for 1 min, submerged in 70 % ethanol for 2 min, treated with 1.4 % sodium hypochlorite (NaClO) for 5 min, and again immersed in 70 % ethanol for 1 min. Subsequently, the samples were washed three times in ddH2O for 1 min. Finally, all leaves belonging to a single sample were tapped dry with fresh tissues, stacked and stamped 5 times with a sterilized cork cutter resulting in 25 leaf discs of 6 mm diameter (706.75 mm^2 total). These discs were transferred into 2 ml tubes (Sarstedt) and frozen immediately at −80 °C until further processing. Control samples were stacked and stamped similarly to the sterilized samples. All tools were sterilized between samples.

2.3. DNA extraction, amplicon library preparation and sequencing

Frozen samples were milled with two 3 mm sterilized steel beads and sterilized glass beads (0.1 mm, 0.5 mm and 1 mm). DNA was extracted using the Chargeswitch gDNA plant Kit (Invitrogen, Thermo Fisher Scientific, Dreieich, Germany) scaled down to 40 % of the manufacturer's recommended reaction volume. To remove polysaccharides and phenolic compounds, the lysis buffer was supplemented with $CaCl_2$ and polyvinylpyrrolidone (average 10.000 MW).

The primer pair ITS1-F and ITS2R (Zhang et al., 2018) was used to amplify the fungal Internal Transcribed Spacer 1 (ITS1) gene region located between 18S and the 5.8S rRNA gene (White, 1990). Primers were fitted with Illumina adapters for multiplexing, using the Nextera XT Index Kit v2 (Illumina, Germany). PCR reactions were performed in a Mastercycler 5341 (Eppendorf, Germany), using a total reaction volume of 25 µl for each sample. Each reaction consisted of 12.5 µl Kappa Hifi Hot start polymerase (Roche), 5 µl of forward and reverse primer with a concentration of 5 µM, 0.5 µl BSA, ddH2O and 2 µl DNA extract.

To increase sensitivity and amplification of a diverse template we used a touchdown PCR (Korbie and Mattick, 2008). The following PCR program was used: initial denaturation at 95 °C for 3 min, 10 cycles of denaturation at 98 °C for 20 s, annealing at 60-50 °C (-1 °C at every cycle) for 30 s, elongation at 72 °C for 45 s, followed by 20 cycles of denaturation at 98 °C for 20 s, annealing at 55 °C for 30 s, elongation at 72 °C for 45 s and a final extension at 72 °C for 10 min.

The resulting amplicon libraries were validated by gel electrophoresis and purified using the Agencourt AMPure XP kit (Beckman Coulter, Krefeld, Germany). Afterwards, dual indices were added during another PCR using the Nextera XT Index Kit v2 (Illumina, Germany). The thermal profile was as follows: initial denaturation at 95 °C for 3 min, 8 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, followed by elongation at 72 °C for 30 s and a final extension at 72 °C for 5 min. The resulting libraries were cleaned from primer fragments using the Agencourt AMPure XP kit (Beckman Coulter, Krefeld, Germany). Finally, the DNA content of each sample was quantified using a Qubit 4 fluorometer (Invitrogen™, Thermo Fisher, Deutschland) and the Qubit 1X dsDNA HS Assay Kit (Invitrogen™, Thermo Fisher, Germany). Fragment sizes and quality of DNA sequencing libraries were determined by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States). Paired-end sequencing of 2 × 300 bp was performed using a MiSeq Reagent kit v3 and 30 % Phiχ on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, United States) at the Sequencing Service at Ludwig-Maximilian University of Munich.

In total, we sequenced two libraries with 386 samples each. Due to low amplification in the first run, samples showing fewer than 5000 raw reads were re-sequenced. Samples failing to meet this threshold after the second run were excluded.

2.4. Bioinformatics workflow

Raw reads generated from the MiSeq platform were demultiplexed and trimmed by the Illumina MiSeq software, using default settings. The resulting fastq files were processed in R (Version 4.2.1) (R Core Team, 2022) with the DADA2 workflow (Version1.16.0) (Callahan et al., 2016). Among the 672 sequenced samples, 515 samples with more than 5000 raw reads were processed further. Initially, raw reads were quality filtered, dereplicated and trimmed according to standard protocol with trimming after 240 bp for the forward reads and 160 bp for the reverse reads. Forward and reverse reads were merged using the DADA2 default overlap of 12 base pairs, followed by removal of chimeric sequences. All steps, from filtering to chimera removal, were conducted separately for each library before the two libraries were combined. Taxonomic assignment was performed using the naive Bayesian classifier method (Wang et al., 2007) implemented in DADA2, with the Unite database (Version 8.3) (Abarenkov et al., 2024). Trophic modes and guilds were subsequently assigned using the package FUNGuildR and the FUNGuild database (Version 1.1) (Nguyen et al., 2016). According to Nguyen et al. (2016), trophic modes classify fungi based on their primary nutritional strategies, which determine how they obtain energy and nutrients (Tedersoo et al., 2014; Nguyen et al., 2016). In contrast, guilds are categories based on the species' ecological roles and resource use strategies (Wilson, 1999; Nguyen et al., 2016). Both classifications allow analyses that are independent of the taxonomy of individual taxa. For example, different fungi may have similar functional roles within an ecosystem, such as wood decay or root-associated fungi, even if they belong to different trophic modes. We extracted the categories "endophyte", "epiphyte" and "mycoparasite" from the FUNGuild database as additional categories complementing the categories of trophic modes. Trophic modes and guilds were further divided based on assignment confidence, retaining only those ranked as "Highly probable" or "Probable" to account for assignment uncertainty. Non-fungal taxa were excluded from further analyses. Overall 6,717,857 reads were assigned to 2990 fungal ITS1 amplicon sequence variants (ASVs) (Callahan et al., 2017).

2.5. Statistical analyses

All statistical analyses were carried out using R (Version 4.2.1) (R Core Team, 2022). The analyses were done separately for each of the three subsets.

1. Non-sterilized samples representing the entire phyllosphere fungal community.
2. Surface-sterilized samples representing the endophytic community sensu stricto.
3. A derived subset, representing true epiphytic taxa, generated by subtracting taxa present in the endophytic subset from the non-sterilized data.

Subsetting was performed using the phyloseq package (McMurdie and Holmes 2013). Epiphytes were determined at the dataset level by filtering the full phyllosphere subset to exclude taxa present in the endophytic subset. We would like to point out that this approach assigns epi-endophytic fungi, i.e. fungi residing both in and on the leaf, to the endophyte and not the epiphyte dataset. Thus, the epiphyte set might to some degree underestimate the epiphyllous fungal community. Alpha-diversity was quantified using the abundance-based Coverage Estimator of species richness (ACE) (Chao and Lee, 1992) and the Shannon diversity index (Shannon, 1948), calculated with the 'estimate_richness' function in phyloseq for all fungi, by kingdom, by phylum,

by trophic mode, and by guild unidentified taxa at the phylum or class level were excluded, while taxa classified as *Incertae sedis* were included as a distinct group, representing unplaced fungi that may belong to known or unknown phyla.

We conducted all analyses for each of the three subsets and provide an overview of used models in the Appendix (Table S30). To test if the phyllosphere, endophytic and epiphytic fungal communities differ in richness and composition (H1). Additionally, we compared surface-sterilized and non-sterilized samples, using a log response ratio (LRR) of ACE calculated for each individual tree. This was derived by dividing the ACE of the entire phyllosphere by the ACE of endophytic taxa and taking the natural logarithm of the resulting ratio. Higher log response ratios indicate greater relative abundance of epiphyllous compared to endophytic taxa, as such the LRR can also be interpreted as calculated relative measure of epiphytic richness. Log response ratios were also calculated for kingdom, phyla, trophic modes and guilds.

Furthermore, beta-diversity, based on Bray–Curtis dissimilarity, was calculated for fungal communities between individual trees, using relative read count. PERMANOVAs, implemented in the 'adonis2' function of the vegan package, was used to test the effects of treatment on fungal community composition.

To determine whether the richness of taxa classified as epiphytes was higher in the phyllosphere, we used a two-proportion Z-test (Sprinthall, 2012) that compares the proportion of epiphytic taxa between the endophyte and the phyllosphere subsets.

Testing if fungal richness is influenced by host identity and neighborhood plant diversity (H2a), mixed effects models were applied to evaluate the influence of tree species richness, host tree species, treatment (sterilized vs. non-sterilized), and their interaction (treatment*tree species), for all three subsets. All models included species composition, plot ID, and tree species as random factors and employed Type III ANOVA for significance testing. All statistical tests were adjusted for false discovery rates (FDR) using the method of Benjamini and Yekutieli (2001). Additionally, conditional and marginal R^2 values were calculated using Nakagawa's method and the performance package (Lüdecke et al., 2021). All models performed using ACE were repeated using Shannon diversity.

This was followed by testing if the fungal community composition is influenced by host identity and neighborhood plant diversity (H2a), using previously calculated Bray–Curtis dissimilarity to test the effects of host tree species and the interaction of host species and treatment, as well as of the host trees' mycorrhizal type and mycorrhizal type diversity on fungal community composition for each subset. PERMANOVAs, implemented in the 'adonis2' function of the vegan package, was used to test the effects of treatment on fungal community composition. Results were visualized using distance-based principal coordinate analyses (dbPCoA).

To assess local neighborhood effects (H2b), additional mixed effects models were used with the presence of each individual neighbor species as predictor for fungal richness, including species composition, plot ID, and tree species as random factors and employed Type III ANOVA for significance testing, as well as FDR p-value adjustment. Dilution effects are detected when fungal richness in a target species decreased with a lower proportion of the target species in the local neighborhood (Fig. 2A). Conversely, spillover effects were identified when fungal richness increased in a target species with increasing proportion of a specific non-target species in the local neighborhood (Fig. 2B).

Testing if fungal community composition is affected by (H3a) the host's mycorrhizal type (AM or EM) or (H3b) the mycorrhizal type composition of the tree community (exclusively AM or EM hosts, or a mix of both host types). We used the previously calculated Bray–Curtis dissimilarity to test the effects of mycorrhizal type and mycorrhizal type composition on fungal community composition for each subset. PERMANOVAs, implemented in the 'adonis2' function of the vegan package, was used to test the effects of treatment on fungal community composition.

3. Results

In total, we found 2990 fungal ITS1 amplicon sequence variants (ASVs) of which 2203 ASVs were detected in the phyllosphere, 1573 ASVs as endophytes, and 1380 ASVs as epiphytes (Fig. 3; Table S2). Discrepancies in the number of ASVs between treatments arose from substantial overlap of ASVs between endophytes and epiphytes, with certain ASVs being exclusive to endophytes or epiphytes.

The distribution of ASVs varied across tree species, ranging from 779 ASVs in *Quercus* to 404 ASVs in *Betula* in the phyllosphere. After leaf-surface sterilization, 50–80 % of phyllosphere ASVs were kept as endophytes in most tree species, with *Sorbus* retaining 92 % and *Fagus* only 30 %. Endophytic ASV richness ranged from 473 ASVs in *Quercus* to 226 in *Prunus*. A similar trend was observed for epiphytes, with 407 ASVs in *Quercus* and 96 in *Sorbus*. Additional information on the taxonomic composition of phyllosphere, endophyte, and epiphyte communities can be found in Fig. S1 and Tables S3, S4, S5, and S6 in the Appendix.

3.1. Effects of tree diversity and local neighborhood on fungal richness

Tree species richness had a significant positive effect on fungal richness in the phyllosphere (p-adjusted = 0.04; $R^2_{cond.} = 0.582$, $R^2_{marg.} = 0.056$), as assessed by the Abundance-based Coverage Estimator of species richness (ACE) (Fig. 4A; Table S7). Positive effects of tree species richness on phyllosphere fungi were observed for the phyla Ascomycota (p-adjusted = 0.04; $R^2_{cond.} = 0.494$, $R^2_{marg.} = 0.053$) and Basidiomycota (p-adjusted = 0.04; $R^2_{cond.} = 0.586$, $R^2_{marg.} = 0.034$), as well as unidentified phyla (p-adjusted = 0.04; $R^2_{cond.} = 0.229$, $R^2_{marg.} = 0.046$).

For trophic modes and guilds, tree species richness positively affected symbiotrophs (p-adjusted = 0.01; $R^2_{marg.} = 0.119$) and patho-sapro-symbiotrophs (p-adjusted = 0.001; $R^2_{marg.} = 0.161$), as well as epiphytes (p-adjusted = 0.002; $R^2_{marg.} = 0.233$) (Table S7). A proportion Z-test confirmed significantly more epiphytic than endophyte taxa in the phyllosphere ($p < 0.001$) (Table S29).

Similarly, we observed a significant effect of tree species richness on epiphyte richness derived by the LRR for the overall fungal community (p-adjusted = 0.043; $R^2_{cond.} = 0.487$, $R^2_{marg.} = 0.067$; Fig. 4B) and Ascomycota (p-adjusted = 0.043; $R^2_{marg.} = 0.121$), while no such effect could be found for the epiphytic community derived by subsetting the phyllosphere community with the endophytic community (Table S23).

In contrast, no significant effects of tree species richness or identity on fungal richness were detected in the endophytic (Fig. 4C, D) (Table S19). However, ASVs unidentified at the phylum level showed a positive association with tree species richness in the endophyte community (p-adjusted = 0.037; $R^2_{marg.} = 0.128$) (Table S19).

There was only limited evidence for spillover effects, with the presence of *Sorbus* in the local neighborhood significantly increasing the richness of highly probable saprotrophs in the phyllosphere of *Fraxinus* (p-adjusted = 0.025; $R^2_{marg.} = 0.194$). Additionally, *Fagus* in the neighborhood increased probable multiple lifestyle taxa in *Quercus* (p-adjusted = 0.023; $R^2_{marg.} = 0.510$) (Table S10). No dilution effects were detected in our analyses (Tables S10, S15, S22).

For additional information regarding the effect tree diversity and local neighborhood on fungal diversity described by the Shannon index see Appendix Tables S8, S11, S12, 14, S17, S18, S20, S23, S24, and S26.

3.2. Effects of leaf-surface sterilization

Fungal richness was significantly reduced in surface-sterilized samples compared to non-sterilized samples across all tree species (p = 0.002; $R^2_{marg.} = 0.613$), confirming the efficacy of the sterilization process (Fig. 4C, D) (Table S27). Responses to leaf-surface sterilization varied among tree species, with *Betula* showing the smallest difference and *Fagus* the largest (Fig. 4D). The log response ratio of ACE revealed a significantly higher ratio of phyllosphere to endophytic fungi in more diverse plots for overall fungal richness (p-adjusted = 0.043; $R^2_{marg.} =$

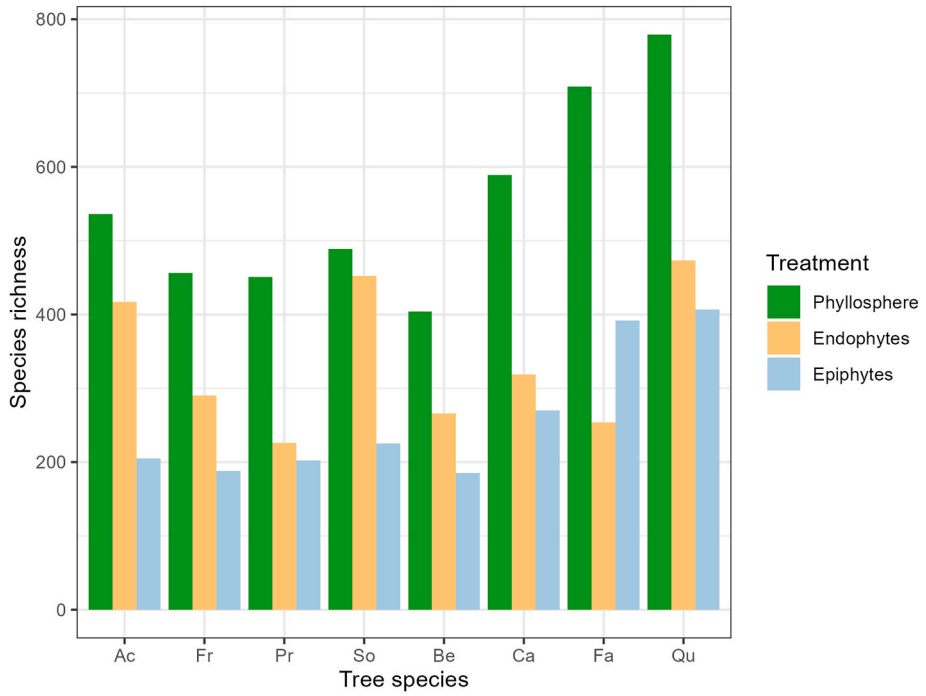

Fig. 3. Barplot showing the number of fungal species for the phyllosphere, endophytes, and epiphytes per tree species. While fungal species richness was directly derived for the phyllosphere and endophyte community, the epiphytic community was calculated as all taxa that are present in the phyllosphere but not as endophytes. As such epiphytes maybe underrepresented in this graph. Ac = *Acer pseudoplatanus*, Fr = *Fraxinus excelsior*, Pr = *Prunus avium*, So = *Sorbus aucuparia*, Be = *Betula pendula*, Ca = *Carpinus betulus*, Fa = *Fagus sylvatica*, Qu = *Quercus petraea.*

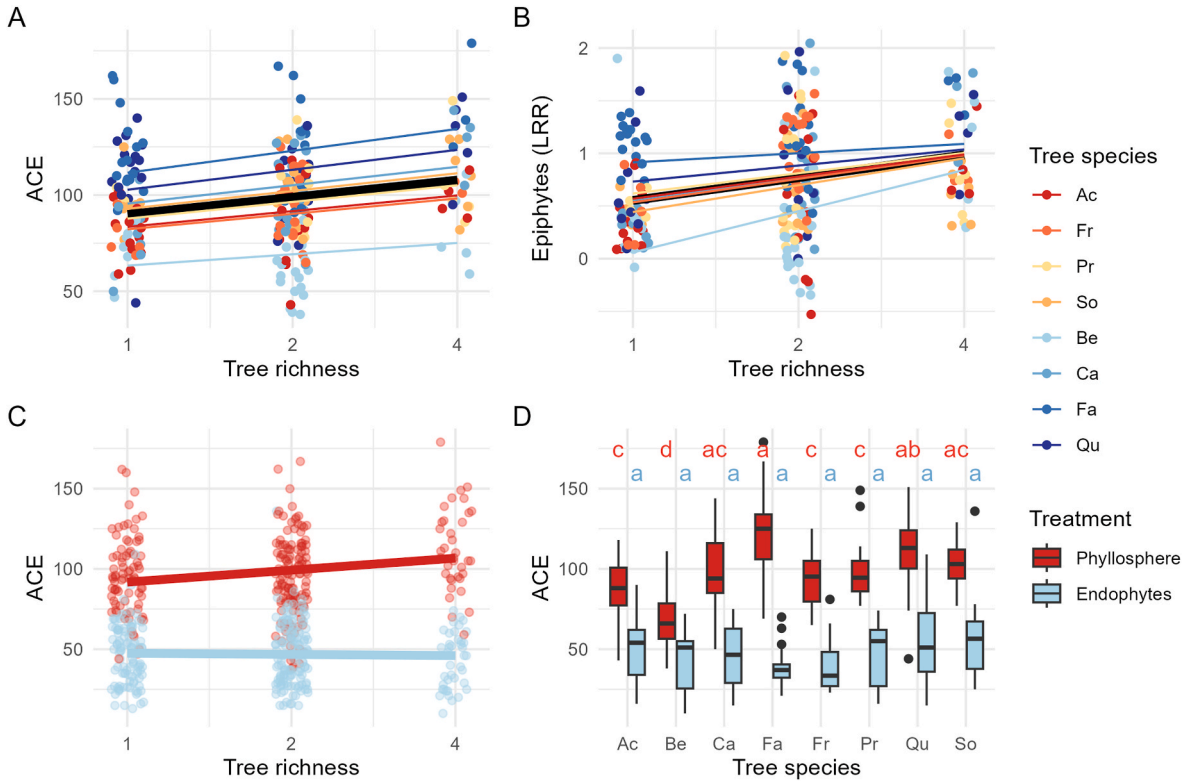

Fig. 4. Abundance-based Coverage Estimator (ACE) of the phyllosphere community as function of tree species richness (A). Endophyte richness expressed by the log response ratio (LRR), which is the natural logarithm of the ratio of phyllosphere to endophyte richness per individual tree (B). Solid lines indicate significant overall slopes (A, B). Colored lines show the random slope effects of the different tree species. Relationship of ACE are shown separately for the phyllosphere (control) and for endophytes (surface sterilized) as a function of tree species richness (C) and of tree species (D). For the statistical tests see Appendix Tables S7 and S11. Ac = *Acer pseudoplatanus*, Fr = *Fraxinus excelsior*, Pr = *Prunus avium*, So = *Sorbus aucuparia*, Be = *Betula pendula*, Ca = *Carpinus betulus*, Fa = *Fagus sylvatica*, Qu = *Quercus petraea.*

0.121) (Fig. 4B) and Ascomycota (p-adjusted = 0.043; $R^2_{cond.}$ = 0.478, $R^2_{marg.}$ = 0.067) (Table S25). For information regarding the effect of surface sterilization on fungal diversity described by the Shannon index see Appendix Tables S26 and S27.

3.3. Fungal community analysis

Distance-based principal coordinate analysis (dbPCoA) revealed distinct clustering of sterilized and non-sterilized samples (Fig. 5), demonstrating the effective separation of endophytic and phyllosphere communities. PERMANOVA results indicated significant effects of tree species identity (p = 0.001; R^2 = 0.494), treatment (p = 0.001; R^2 = 0.13), and interaction (p = 0.001; R^2 = 0.032) on fungal community composition (Table S28). Similar patterns of tree species identity were observed for endophytes (p = 0.001; R^2 = 0.614) and epiphytes (p = 0.001; R^2 = 0.093) (Table S28).

3.4. Effect of mycorrhizal type and mycorrhizal type mixtures

Both the mycorrhizal type of the host tree and the mixture of mycorrhizal types at the plot level exerted only minor effects on phyllosphere fungal communities. However, PERMANOVA analyses identified significant effects of both mycorrhizal type and mycorrhizal diversity on fungal community composition in the phyllosphere and epiphytes. In the phyllosphere, mycorrhizal type (p = 0.001; R^2 = 0.098) and mycorrhizal diversity (p = 0.028; R^2 = 0.008) explained 9 % and 0.8 % of the variance of fungal community composition, respectively (Table S28). Fungal community composition clearly differed between the host trees' mycorrhizal types, with AM trees forming a single cluster while among EM trees Betula formed a separate group (Fig. 5B). In contrast, mycorrhizal type diversity did not result in a clear separation of fungal communities (Fig. 5C). In contrast, for epiphytes mycorrhiza type (p = 0.001; R^2 = 0.014) and mycorrhizal diversity (p = 0.002; R^2 = 0.007) accounted for 1.4 % and 0.7 % of the variance, respectively. For endophytes, only tree mycorrhizal type of the host tree showed a significant effect (p = 0.001; R^2 = 0.082), explaining 8.2 % of the variance (Table S28).

4. Discussion

Our findings demonstrate that leaf-surface sterilization is an effective method for splitting foliar fungal communities into phyllosphere, endophytic, and epiphytic fractions, enabling detailed interpretation of host tree identity and neighborhood effects for each community. These results support our first hypothesis, as excluding epiphyllous fungi via surface sterilization significantly altered fungal phyllosphere community composition.

Previous studies by Guerreiro et al. (2018) and Yao et al. (2019) reported that leaf-surface sterilization reduced fungal operational taxonomic units (OTU) counts for endophytes from 66 % to 40 % of total fungal OTUs from the phyllosphere. Conforming with these findings, our results showed that 53 % of ASVs from non-sterilized samples were

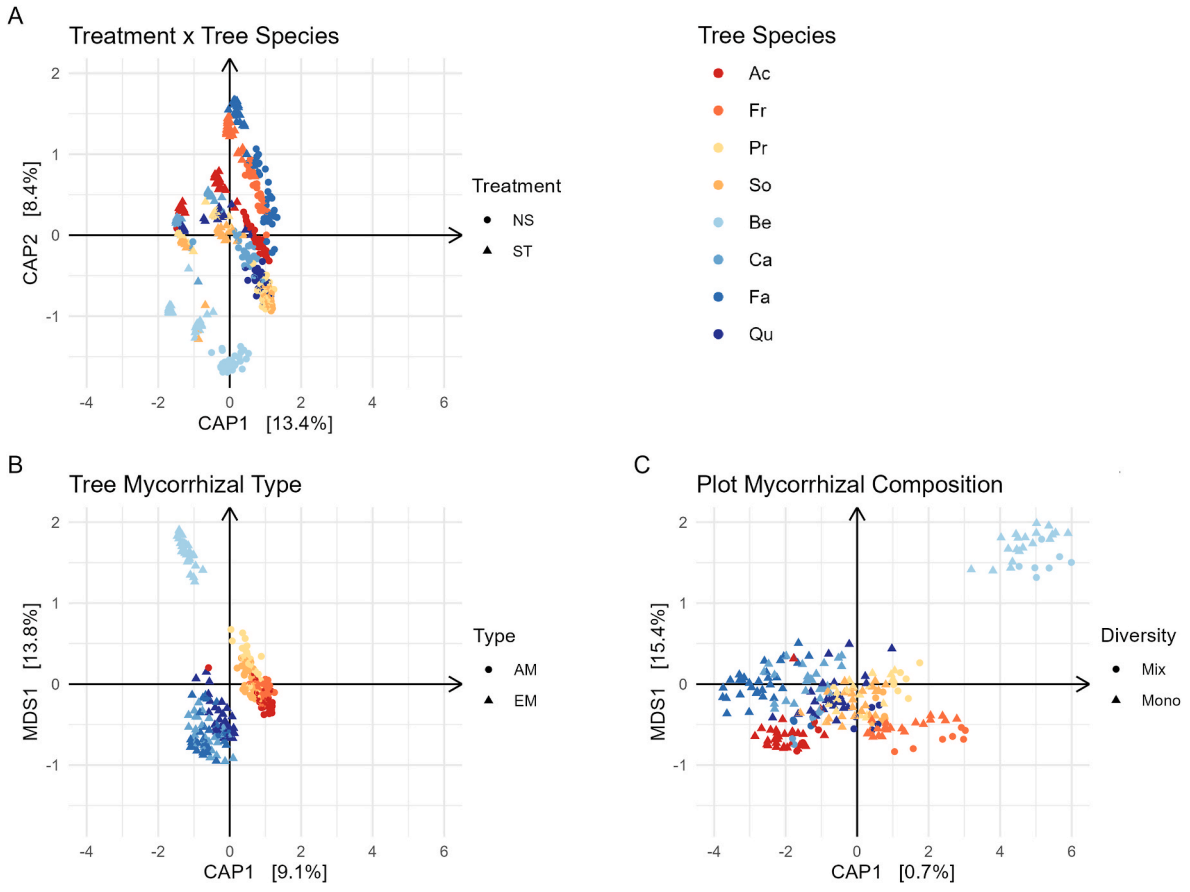

Fig. 5. Distance-based principal coordinate analysis (dbPCoA) of fungal community composition, using three different sets of constraining variables. A) Effect of the combination of treatment (NS = control, ST = surface sterilization) and tree species as constraining variables. According to a permutation test treatment, tree species and the interaction of treatment and tree species were significant (p < 0.001). B) Effect of the host tree mycorrhizal type, either arbuscular mycorrhiza (AM) or ectomycorrhiza (EM), as constraining variable (p < 0.001). C) Effect of the plot mycorrhizal composition, either monotypic (mono) or mixed mycorrhizal types (mixed), as constraining variable (p < 0.028). Axes determined by constraining variables are referred to as CAP (canonical analysis of principal coordinates), while unconstrained axes are referred to as MDS (multidimensional scaling). For abbreviation of tree species names see Fig. 3. For the statistical tests see Appendix Table S28.

retained after sterilization, indicating that roughly half of the ASVs were removed. This pattern was consistent across all tree species, suggesting that epiphyllous taxa constitute approximately half of the phyllosphere fungal community. These findings support the general view that leaf surfaces act as efficient fungal traps, capturing spores and fungal organisms from the surrounding environment (Sgrigna et al., 2020). While ungerminated spores likely represent the majority of the epiphyllous fungal community, yeasts and pathogens may use the leaf surface as a suitable microhabitat, what aligns with previous fungi reports identifying biotrophic and saprotrophic epiphyllous fungi (Bauer et al., 2006; Quan et al., 2020; Marasinghe et al., 2023).

Our second hypothesis, that fungal richness depends on the host species' identity and on neighborhood tree richness was only partially confirmed. Host species was a key driver of fungal richness and community composition for all community fractions, endophytes, epiphytes, and the entire phyllosphere. However, neighborhood diversity influenced fungal richness only in the phyllosphere and for epiphytes derived by the LRR. Thus, hypothesis 2a was supported for the full phyllosphere and epiphyte communities but not for endophytic fungi. The stronger response of epiphytes to local neighborhood diversity may stem from their passive accumulation of spores from neighboring trees. This explanation aligns with those of Saadani et al. (2021), who observed increased phyllosphere fungal richness with a larger neighborhood diversity. Conversely, studies excluding epiphyllous taxa focusing on the endophytic community found no such effects (Griffin et al., 2019; Kambach et al., 2021). The accumulation of fungal spores may increase with neighborhood diversity due to the diversity of sporulating fungi hosted by different tree species. Furthermore, forest stands with higher tree species richness exhibit greater stand structural complexity, providing a higher variation in resources, and thus, niche opportunities for fungi (Ishii et al., 2004; Ali et al., 2016; Madi et al., 2020). This structural complexity may also enhance air-current resistance, resulting in a stronger filtering effect (Sgrigna et al., 2020; Ray et al., 2023). Notably, the positive relationship between tree species richness and phyllosphere fungal richness was particularly pronounced for epiphytic fungi. This suggests that increasing tree species richness actively creates microhabitats for biotrophic epiphyllous taxa complementing passive spore accumulation. Experimental manipulation is needed to quantify the relative importance of these different mechanisms.

The observed patterns may also reflect contributions from epiphyllous pathogenic fungi. Hantsch et al. (2014a) found strong positive relationships between fungal pathogen richness and local host richness, driven by host-specific pathogen species. Such patterns are likely governed by dilution effects (Civitello et al., 2015; Wang et al., 2023) rather than spillover effects (Hantsch et al., 2013; Saadani et al., 2021).

Our findings offer limited support for hypothesis 2b, as tree-species-specific effects were weak and mostly undetectable, suggesting that dilution and spillover effects were of minor importance in this study, thus contrasting the results reported by Field et al. (2020) and Hantsch et al. (2014a). However, the high number of different neighbor species in our study involves the disadvantage of limited statistical power to detect dilution and spillover effects, which leaves the possibility that they exist but are subtle.

The strong host-species-specific effects observed in fungal endophyte and epiphyte community composition were consistent with previous studies (Darcy et al., 2020). These effects may reflect phylogenetic relatedness, as similarity in endophyte composition correlates with host phylogeny (Rutten et al., 2021). However, testing phylogenetic effects would require a broader host species range than present in this study. Functional traits of leaves, such as specific leaf area and leaf carbon content, may also play a role (Kembel and Mueller, 2014; González-Teuber et al., 2020; Wang et al., 2023).

We hypothesized that the mycorrhizal status of tree species influences the composition of fungal phyllosphere communities. The underlying mechanism would either be the preference of a tree species for specific fungal groups or through the direct involvement of mycorrhiza in shaping the endophytic community via chemical signaling (Novas et al., 2009; Debray et al., 2022). Our findings indicate that the preferred mycorrhiza type of the host tree significantly influenced the overall fungal community composition in the phyllosphere, including endophytes and epiphytes, thus supporting our hypothesis 3a.

Similarly, we found support for hypothesis 3b, which posited that fungal community composition is influenced by the mycorrhizal type composition of the surrounding tree community for the whole phyllosphere and epiphytic community.

These results confirm our third hypothesis that tree mycorrhizal type and the mixing of mycorrhizal types affect the fungal community composition of leaves. It is plausible that the observed effects were driven more by species identity effects of particular AM and EM trees in the mixtures than by mixing effects of different mycorrhizal types. To disentangle these species identity effects from genuine mycorrhizal mixing effects, future studies would need experimental designs explicitly aimed at separating these mechanisms.

5. Conclusion

Our study highlights the effectiveness of leaf-surface sterilization as a tool to distinguish between the phyllosphere, endophytic, and epiphytic fungal communities and to investigate their responses to tree species richness and related factors. Distinct fungal assemblages were observed across all fungal community types and host tree species. Importantly, the richness of full phyllosphere and epiphytic communities were significantly influenced by tree species richness, whereas strictly endophytic taxa showed no notable response. This suggests that local neighborhood effects are not driven solely by the dispersal of fungal spores to leaf surfaces or by fungi confined to the leaf interior but are primarily associated with epi-endophytic taxa, that inhabit both the leaf surface and the leaf interior.

Further studies should focus on this group of epi-endophytic fungi by incorporating analyses of the surface-washed fractions, offering a more comprehensive understanding of their ecological role. Additionally, our findings provide limited evidence for the influence of host mycorrhizal type and mycorrhizal type mixtures on fungal community composition. However, we acknowledge that variation in fungal assemblages across individual leaves within a tree introduces an additional layer of complexity, potentially modulating the responses of the phyllosphere microbiome to these factors.

CRediT authorship contribution statement

Michael Köhler: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Georg Hähn:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Maarten Kanitz:** Writing – review & editing, Investigation. **Olga Ferlian:** Writing – review & editing. **Nico Eisenhauer:** Writing – review & editing. **Tesfaye Wubet:** Writing – review & editing, Supervision, Conceptualization. **Helge Bruelheide:** Writing – review & editing, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Data availability

The amplicon data generated for this study can be found in Sequence Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI) under bioproject number PRJNA1120618. Additionally, the phyloseq-object including sampling data from the MyDiv experiment can be found in the MyDiv database under doi.org/10.25829/2QXM-DK15.

Code availability

Code available at doi.org/10.5281/zenodo.15341135.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2025.101440.

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