



Article

Fungal Microbiome Structure Across Phyllosphere Compartments in Intensively Managed *Eucalyptus cinerea* for Cut Foliage Production

Tomás Byrne ¹ and Dheeraj Singh Rathore ^{2,*} ¹ Teagasc, Forestry Development Department, Ashtown, D15 DY05 Dublin, Ireland; tomas.byrne@teagasc.ie² Teagasc, Forestry Development Department, Oak Park Research Centre, R93 XE12 Carlow, Ireland

* Correspondence: dheeraj.rathore@teagasc.ie

Abstract

Fungal communities associated with the phyllosphere can influence plant health, stress responses, and disease dynamics in managed crop systems. However, limited information is available on fungal microbiome structure across phyllosphere compartments of *Eucalyptus cinerea* cultivated for cut foliage production. In this study, fungal communities (including epiphytic and endophytic fungi) associated with leaf, stem, and bark tissues of intensively managed *E. cinerea* grown in Ireland were characterised using ITS amplicon sequencing. Samples were collected from five trees, with tissues pooled by compartment to generate 15 biological samples. Following quality control and denoising, 405 fungal amplicon sequence variants (ASVs) were retained for analysis. Observed richness, Shannon and Simpson indices, and Faith's phylogenetic diversity differed among compartments, with bark exhibiting higher values than leaf and stem tissues ($p < 0.05$). PERMANOVA analysis indicated that both compartment ($R^2 = 0.239$, $p = 0.002$) and tree identity ($R^2 = 0.451$, $p = 0.002$) significantly influenced fungal community composition. Bark communities were dominated by *Diaporthe* (52.9%), *Peniophora* (12.8%), and *Talaromyces* (10.4%), whereas leaf and stem communities were characterised primarily by *Vishniacozyma* and *Sporobolomyces*. Differential abundance analysis identified 26 and 23 differentially abundant ASVs between bark and leaf, and bark and stem tissues, respectively, whereas no significant differences were detected between leaf and stem communities. Weighted UniFrac analyses further revealed separation of bark-associated communities from photosynthetic tissues. These findings demonstrate compartment-associated variation in fungal community structure within the phyllosphere of managed *E. cinerea* and highlight the importance of considering both host-level and tissue-level effects in plant microbiome studies. This study provides a baseline assessment of fungal assemblages associated with commercially managed *Eucalyptus* under Irish growing conditions and supports future investigations into the functional significance of these microbial communities for plant health and resilience.



Academic Editor: Alessandro Vitale

Received: 15 May 2026

Revised: 2 July 2026

Accepted: 5 July 2026

Published: 7 July 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](#) license.

Keywords: phyllosphere microbiome; *Eucalyptus cinerea*; fungal community structure; ITS amplicon sequencing; compartmentalisation; phylogenetic diversity; plant–microbe interactions; managed cropping systems

1. Introduction

The genus *Eucalyptus* is globally cultivated as a major source of timber, pulpwood, charcoal production, essential oils, and increasingly for ornamental and cut foliage markets [1].

Among these, *Eucalyptus cinerea* has gained commercial significance for the production of cut foliage branches due to its distinctive juvenile leaf morphology and high market demand in the floriculture industry [2]. In Ireland, *E. cinerea* is increasingly cultivated as a high-value cut foliage crop, with expanding interest in its commercial production under managed systems [3]. Under intensive production systems, maintaining crop uniformity, productivity and quality is vital, and such traits are influenced by several factors, including host genetics background, agronomic practices, soil physicochemical properties, as well as associated microbial communities [4]. In cut foliage production specifically, phyllosphere fungal communities associated with above-ground plant tissues may directly impact product quality through their potential roles in surface colonisation, postharvest deterioration, foliar disease development, and leaf senescence dynamics; yet this dimension of crop health management remains largely unexplored in *Eucalyptus* species.

As a non-native species cultivated under temperate Irish conditions, *Eucalyptus* is exposed to a range of biotic and abiotic stresses, including foliar pathogens, environmental variability, and management-related disturbances [3]. Fungal communities associated with plant tissues, including phyllosphere and endophytic fungi, have been shown to influence plant health, stress tolerance, and disease dynamics across a range of woody and perennial plant species [5–7]. Understanding the composition and structure of these communities is therefore important for establishing baseline knowledge relevant to improving crop resilience and quality in managed production systems.

The aerial surfaces of plants, collectively referred to as the phyllosphere, harbour a complex microbial habitat that hosts diverse fungal communities, including epiphytes, endophytes, and latent pathogens [8]. In this study, the term phyllosphere is used in its broad sense to encompass above-ground plant-associated habitats, including leaf, stem, and bark tissues. Fungal communities associated with these tissues can contribute to nutrient cycling, modulation of host stress responses, and interactions with pathogens [6]. In woody perennial crops, such interactions are particularly important due to prolonged exposure to environmental pressures and cumulative microbial colonisation over time.

Microbial community assembly in the phyllosphere is influenced by host genotype, environmental conditions, and tissue-specific microhabitats. Plant compartments such as the leaf, stem, and bark differ in structure, nutrient availability, degree of lignification and exposure to environmental fluctuations, all of which can drive distinct microbial assemblages [9]. Bark represents a structurally distinct microhabitat that may favour fungi associated with lignified substrates and long-term colonisation, whereas leaves and young stems typically support more dynamic and diverse communities [10]. In addition, variation among individual plants can contribute significantly to microbiome composition, even under relatively uniform management conditions.

Despite increasing interest in plant-associated microbiomes, there remains limited understanding of fungal community structure across phyllosphere compartments of *E. cinerea* grown under commercial management, particularly in temperate regions such as Ireland. Previous microbiome studies in other plant systems have shown that microbial assemblages may vary among tissue types and individual host plants, but there remains a specific knowledge gap regarding how fungal communities are organised across leaf, stem, and bark compartments of *E. cinerea* under Irish growing conditions. This information is important for understanding the microbial baseline of a commercially relevant crop and for guiding future studies on plant health, postharvest quality, and microbiome-informed crop management.

We hypothesised that the fungal community composition would differ among leaf, stem, and bark compartments due to differences in tissue structure and microenvironment, and that bark would harbour a distinct fungal assemblage compared with photosynthetic

cally active tissues. In this study, we characterised the fungal microbiome associated with the phyllosphere of managed *E. cinerea* by comparing fungal communities across leaf, stem, and bark compartments. Using high-throughput ITS amplicon sequencing, we (i) assess patterns of alpha and beta diversity, (ii) evaluate the relative influence of plant compartment and host identity on community structure, and (iii) examine taxonomic and phylogenetic differences among fungal assemblages. This work provides a baseline understanding of fungal community structure in a commercially relevant *Eucalyptus* production system and offers insights into the potential role of phyllosphere fungi in plant health and resilience.

2. Materials and Methods

2.1. Plant Sampling

Samples were collected on the same day from a commercially managed *E. cinerea* plantation located in County Dublin, Ireland (53.513° N, 6.204° W) in July 2024. The *E. cinerea* crop was established in 2019 and managed under intensive production conditions, including irrigation, fertilisation, and weed control using herbicides. Trees were cultivated under standard commercial cut foliage production practices and were of similar age and management history.

Five trees were randomly selected across three production fields on the site, with inter-tree distances ranging from approximately 15 to 200 m to capture spatial variation while maintaining independence among sampled individuals. From each tree, leaf, stem, and bark tissues were collected, generating three compartment-specific samples per tree ($n = 15$ samples in total).

For each tree, six leaves, six stem sections (approximately 2 cm in length), and six bark pieces were collected from multiple canopy positions and pooled to generate a representative composite sample for each compartment. Pooling was employed to reduce the influence of localised within-canopy variation and to provide sufficient biomass for DNA extraction. While this approach precludes assessment of within-tree spatial heterogeneity, it was considered appropriate for the primary objective of comparing fungal communities among plant compartments.

Samples were placed on ice immediately after collection, transported to the laboratory and stored at -80°C until further processing. Surface sterilisation was not performed prior to DNA extraction, and therefore the resulting fungal community profiles represent a mixture of epiphytic and endophytic fungi associated with each compartment.

2.2. DNA Extraction, Library Prep and Sequencing

Total genomic DNA was extracted from pooled leaf, stem, and bark samples using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. DNA quality and concentration were assessed prior to library preparation.

The fungal ITS1 region was amplified using the primers ITS5-1737F (5'-GGAAGTAA-AAGTCGTAACAAGG-3') and ITS2-2043R (5'-GCTGGTTCATTCATCGATGC-3') [11], which amplify the ITS region spanning the ITS1 spacer, the 5.8S rRNA gene and the ITS2 spacer. PCR amplification and library preparation were performed by Novogene Co., Ltd. (Cambridge, UK) following their standard amplicon sequencing protocols.

Negative controls were included for library preparation. However, the controls failed to produce detectable amplification products and were therefore not included in sequencing. No fungal amplification was detected in the negative controls.

Sequencing was performed on an Illumina NovaSeq platform (Illumina, San Diego, CA, USA) using paired-end chemistry (PE150). The sequencing run was designed to achieve a target depth of approximately 50,000 reads per sample; however, the actual sequencing depth

varied among samples due to normal variation in cluster generation and demultiplexing efficiency. The achieved sequencing depth for each sample is reported in Table 1.

Table 1. Sequencing summary pre- and post-quality control (QC) by compartment. Number of biological replicates (trees) per compartment, $n = 5$.

Compartment	Raw Reads ($\times 10^3$)	Cleaned Reads ($\times 10^3$)	% Retained	Merged Reads (Post-Chimera Removal)
Bark ($n = 5$)	100.0 $\pm$ 17.5	98.0 $\pm$ 16.4	95.0 $\pm$ 1.7	12,178 $\pm$ 3867
Leaf ($n = 5$)	110.8 $\pm$ 5.2	108.9 $\pm$ 4.5	98.0 $\pm$ 0.6	21,069 $\pm$ 1917
Stem ($n = 5$)	106.4 $\pm$ 4.2	102.9 $\pm$ 3.9	97.6 $\pm$ 0.7	20,561 $\pm$ 2159

2.3. Sequence Processing and Bioinformatic Analysis

Raw paired-end sequencing reads were quality filtered and adapter trimmed using Fastp (v1.1.0) [12] prior to downstream processing. Sequence denoising, error correction, paired-end merging, and chimera removal were performed using the DADA2 pipeline implemented within QIIME2 (v2024.2) [13,14]. Reads were truncated at 230 bp based on quality score profiles ($Q \geq 30$). Amplicon sequence variants (ASVs) were inferred directly from sequence data without clustering into operational taxonomic units (OTUs).

Taxonomic assignments were performed using a Naïve Bayes classifier implemented in QIIME2 (feature-classifier plugin) and trained against the UNITE fungal ITS reference database (DOI: 10.15156/BIO/3301241) [15]. Non-fungal ASVs, as identified by the UNITE classification (Kingdom $\neq$ Fungi), were removed prior to downstream analysis. No additional post-DADA2 abundance threshold was applied. A reduction in low-abundance noise was achieved through DADA2's denoising algorithm, which removes many sequencing artefacts during ASV inference.

To support phylogeny-informed analyses, a multiple sequence alignment of the filtered ASVs was generated using MAFFT (v7.525) [16]. A phylogenetic tree was constructed using FastTree (v2.1.11) [16,17], and branch lengths were standardised using Grafen's method prior to the calculation of Faith's phylogenetic diversity (PD) and Weighted UniFrac distances. Given the known limitations of ITS alignments across highly divergent fungal taxa, these phylogeny-based analyses were considered complementary to taxonomic and abundance-based community analyses.

All bioinformatic processing was conducted using QIIME2 (v2024.2), and downstream statistical analyses were performed in R (v4.5.0). Complete analysis scripts and workflows are publicly available through the project's GitHub (v3.6.2) repository (see Data Availability Statement).

2.4. Statistical Analysis

All statistical analyses were conducted in R (4.5.0) [18]. Data processing and visualisation were performed using the packages phyloseq (1.52.0) [19], dplyr (1.1.4), and ggplot2 (3.5.2) [20].

Rarefaction curves were generated using the vegan package (2.7-1) [21] to assess sequencing depth and sampling completeness. Alpha diversity was evaluated using observed richness, Shannon, and Simpson indices [22–28]. Differences in alpha diversity among compartments were tested using pairwise Dunn's tests with Bonferroni correction [27].

Beta diversity was assessed using Bray–Curtis dissimilarity and visualized using principal coordinates analysis (PCoA). For beta-diversity analyses, samples were proportionally normalized (relative abundance transformation: each ASV count divided by the sample total) prior to distance matrix calculation. This approach preserves all sequenced reads and avoids the information loss associated with rarefaction, consistent with current recommendations for microbiome analyses [19]. Non-metric multidimensional scaling

(NMDS) was used as a supplementary ordination approach [28–30]. Distance measures including Bray–Curtis (abundance-weighted) and Euclidean distances were compared to assess sensitivity of ordination results to metric choice [28,31]. Euclidean distance was included as a reference metric for evaluating the Z-score normalized heatmap clustering; its application to raw count data for community comparisons is acknowledged as less ecologically appropriate than dissimilarity-based metrics for sparse compositional data. Permutational multivariate analysis of variance (PERMANOVA) was performed using the *adonis2* function in the *vegan* package to assess the effects of compartment and tree identity on community composition [32]. Homogeneity of multivariate dispersions was tested prior to PERMANOVA interpretation using *betadisper* (v2.7).

Phylogeny-informed diversity metrics were also calculated. Faith's phylogenetic diversity (picante 1.8.2; PD) was estimated using the *picante* package (v1.8.2), and weighted UniFrac (phyloseq) distances were used for phylogenetic beta diversity. Although ITS-based phylogenetic inference is less reliable than marker genes used in bacterial studies due to uneven evolutionary rates and alignment challenges across fungal lineages, MAFFT alignments of ITS sequences have been used in phylogeny-informed diversity analyses with recognised limitations. We retained phylogenetic metrics to complement abundance-based approaches, while acknowledging that absolute phylogenetic distances should be interpreted with caution [33,34].

Taxonomic composition was summarised at the genus level using relative abundance plots. Phyloseq and ggplot2 were used to create mean relative abundance graphs per compartment. Heatmaps of the most abundant taxa were generated following Z-score normalisation and hierarchical clustering based on Euclidean distances [35]. Differential abundance analysis between compartments was performed using DESeq2 (1.48.1) [36]. Raw ASV count data were used as input without prior normalization, as DESeq2 applies its own geometric mean-based size factor normalization internally. A design formula of '*~ tree_no + compartment*' was used, with pairwise contrasts performed between each compartment combination. Dispersion was estimated using the default DESeq2 shrinkage estimator. Significance thresholds were set at a false discovery rate (FDR) < 0.05 and a baseMean > 10; the baseMean threshold was applied to exclude low-count ASVs for which fold-change estimates are unreliable.

Indicator species analysis was performed using the *indicspecies* package (1.7.12) to identify fungal genera and species significantly associated with individual compartments. The *multipatt* function was used with 999 permutations to assess the significance of indicator values, with significance set at $p \leq 0.05$.

3. Results

3.1. Sequencing Quality

Illumina sequencing generated high-quality ITS amplicon datasets across all 15 samples from leaf, stem, and bark tissues collected from five individual trees. The mean raw sequencing depth ranged from $100.0 \pm 17.5 \times 10^3$ reads for bark samples to $110.8 \pm 5.2 \times 10^3$ reads for leaf samples (Table 1). Following quality filtering, 95.0–98.0% of reads were retained, resulting in mean cleaned read counts of $98.0 \pm 16.4 \times 10^3$, $108.9 \pm 4.5 \times 10^3$, and $102.9 \pm 3.9 \times 10^3$ reads for bark, leaf, and stem samples, respectively.

After paired-end merging, denoising, and chimera removal, mean read counts per sample were $12,178 \pm 3867$ for bark, $21,069 \pm 1917$ for leaf, and $20,561 \pm 2159$ for stem tissues (Table 1). A total of 405 fungal amplicon sequence variants (ASVs) were retained following taxonomic assignment and removal of non-fungal sequences which were used for all subsequent analyses.

Rarefaction curves showed continued accumulation of ASVs across the observed sequencing depth range and did not reach a clear asymptote in any compartment (Figure 1). Bark samples generally exhibited higher observed richness than leaf and stem samples throughout the rarefaction curves, indicating greater detectable fungal richness in bark-associated communities.

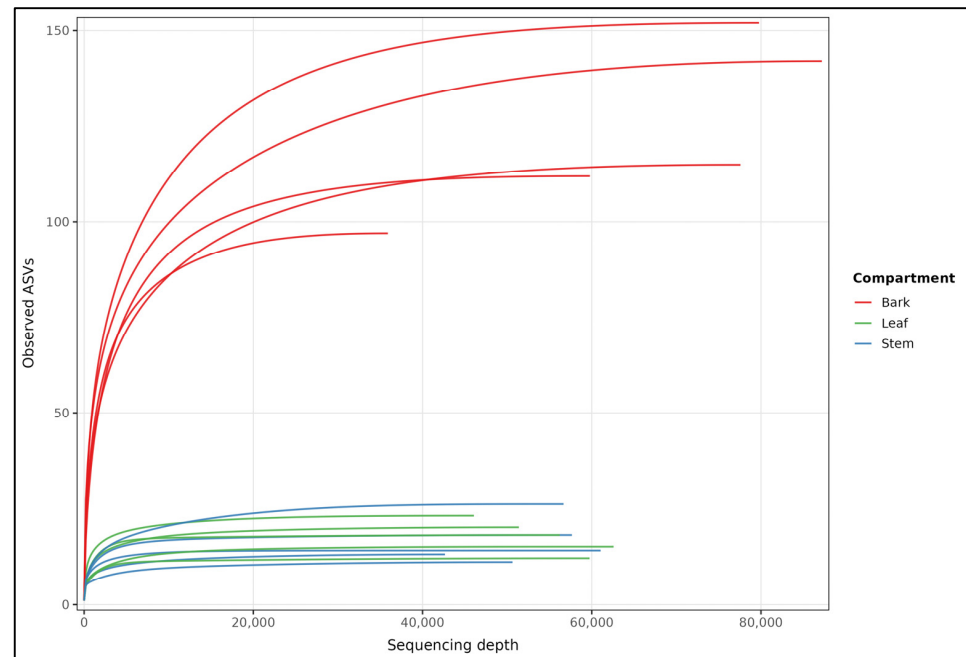


Figure 1. Rarefaction curves for different compartments of *Eucalyptus cinerea* phyllosphere, showing the relationship between sequencing depth and observed ASV richness.

3.2. Fungal Diversity

Alpha diversity varied among plant compartments, although the magnitude and significance of these differences depended on the diversity metric examined (Figure 2). Observed richness differed significantly among compartments, with bark samples exhibiting higher observed fungal richness than both leaf and stem samples (adjusted $p = 0.027$ for each). Leaf and stem samples did not differ significantly from one another (adjusted $p = 1.0$).

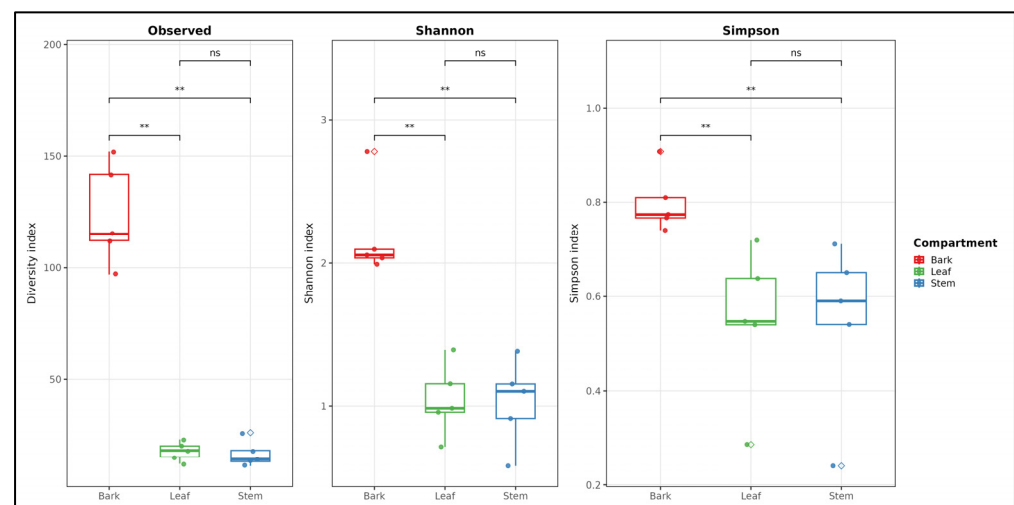


Figure 2. Alpha diversity measures of differing compartments of *Eucalyptus cinerea* phyllosphere. Pairwise differences between compartments were assessed using Dunn's test. Asterisks denote significance (** $p < 0.005$), and ns indicates non-significant differences.

In contrast to the richness pattern, bark samples exhibited significantly higher Shannon and Simpson diversity than leaf and stem tissues (adjusted $p = 0.027$ for each pairwise comparison; Figure 2), whereas leaf and stem communities did not differ significantly from one another (adjusted $p = 1.0$). This indicates that in addition to harbouring more detectable taxa, bark communities were also more diverse when accounting for both richness and evenness. Beta diversity analyses were conducted to assess differences in fungal community composition among compartments and individual trees. Principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarities showed partial separation of samples according to both compartment and tree identity (Figure 3). The first two axes explained 57.6% of the total variation (PC1 = 30.2%, PC2 = 27.4%), while the distribution of variance across ordination axes is provided in Figure S1. Non-metric multidimensional scaling (NMDS) was explored as a supplementary ordination approach, with Shepard plot analysis indicating a strong correspondence between ordination distances and observed dissimilarities (non-metric fit $R^2 = 0.982$; linear fit $R^2 = 0.900$) (Figure S2).

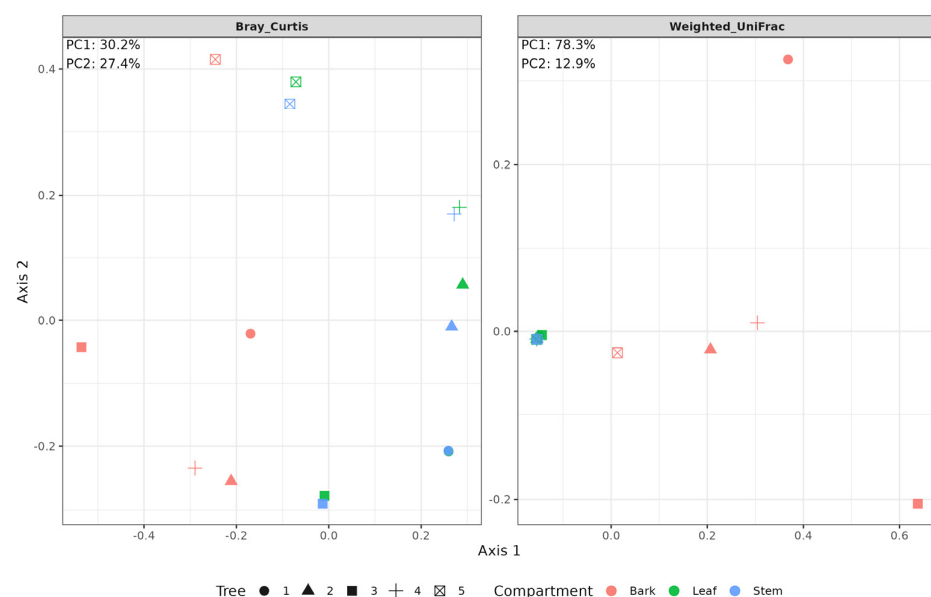


Figure 3. Beta diversity of fungal communities across compartments of *Eucalyptus cinerea* phyllosphere. (**Left panel**): PCoA of Bray–Curtis dissimilarities of ASV relative abundances; the first two axes explain 57.6% of the variance (PC1 = 30.2%, PC2 = 27.4%). (**Right panel**): PCoA of weighted UniFrac distances normalized by library size; the first two axes explain 91.2% of the variance (PC1 = 78.3%, PC2 = 12.9%). Points are coloured by compartment, and shapes denote individual trees.

Prior to PERMANOVA, homogeneity of multivariate dispersion was assessed using betadisper. Dispersion did not differ significantly among compartments ($F_{2,12} = 0.716$, $p = 0.508$; group dispersions: Bark = 0.445, Leaf = 0.343, Stem = 0.323), supporting the validity of subsequent PERMANOVA interpretation.

PERMANOVA based on Bray–Curtis dissimilarities indicated that both compartment and tree identity significantly influenced fungal community composition (Table 2). Tree identity explained the largest proportion of variation ($R^2 = 0.451$, $F = 2.900$, $p = 0.002$), whereas compartment also contributed significantly to community structuring ($R^2 = 0.239$, $F = 3.073$, $p = 0.002$).

Phylogeny-informed beta diversity based on weighted UniFrac distances showed a different pattern of community organisation (Figure 3). The first two axes of the PCoA explained 91.2% of the total variance (PC1 = 78.3%, PC2 = 12.9%). In this ordination, bark samples were positioned separately from leaf and stem samples along the primary axis, whereas leaf and stem samples showed greater overlap.

Table 2. PERMANOVA of factors affecting community structure of *Eucalyptus cinerea* phyllosphere. Measures were based on Bray–Curtis dissimilarities with 999 permutations run. R^2 is the effect size, and F represents a pseudo- F statistic of the ratio of between- and within-group variation.

Factor	Df	Sum of Squares	R^2	F	p -Value
Compartment	2	0.7368	0.239	3.073	0.002
Tree	4	1.3905	0.451	2.900	0.002
Residual	8	0.9589	0.311		
Total	14	3.0862	1		

3.3. Taxonomic Compositions

At the genus level, fungal communities exhibited distinct taxonomic profiles among plant compartments (Figure 4). Bark samples were dominated by the genus *Diaporthe*, which accounted for 52.9% of the mean relative abundance, followed by *Peniophora* (12.8%) and *Talaromyces* (10.4%). Additional genera, including *Paraphaeosphaeria*, *Niesslia* and *Neodevriesia*, were present at lower relative abundances (approximately 4% each).

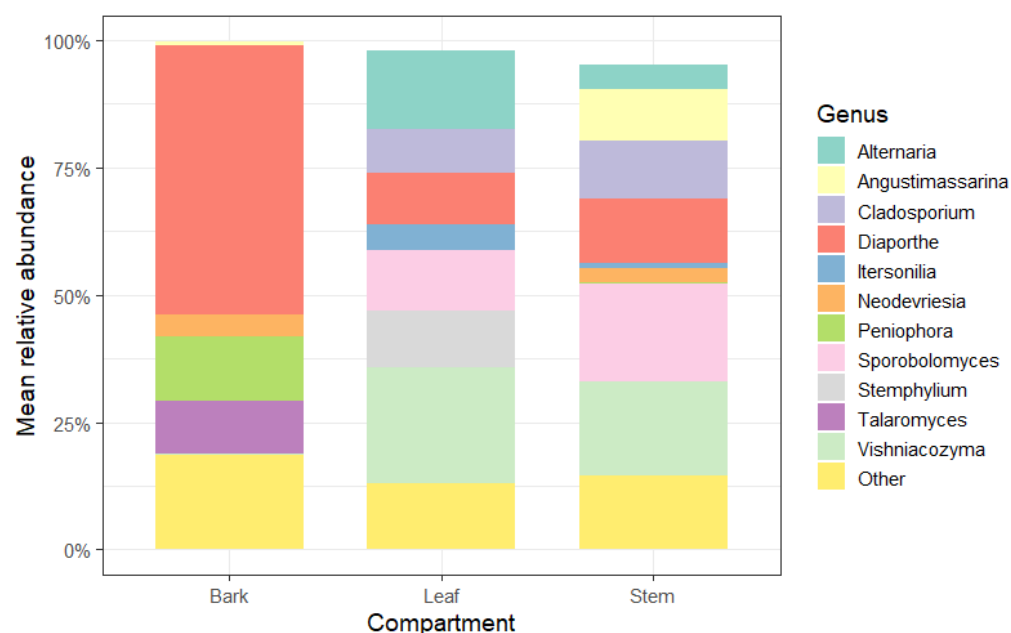


Figure 4. Genus-level relative abundances. Bar plots show mean relative abundance (%) of the top 11 most abundant genera within each compartment, calculated as the proportion of reads assigned to each genus relative to total fungal reads within that compartment.

In contrast, leaf and stem communities showed less pronounced dominance by any single genus, though their overall diversity was lower than that of bark (Figure 2). Leaf samples were characterised by higher relative abundances of *Vishniacozyma* (22.8%), *Alternaria* (15.4%), *Sporobolomyces* (12.1%), and *Stemphylium* (11.0%), together with moderate contributions from *Diaporthe* (10.1%) and *Cladosporium* (8.6%). Stem samples showed a similar composition, with *Sporobolomyces* (19.2%) and *Vishniacozyma* (18.4%) as the most abundant genera, followed by *Diaporthe* (12.6%), *Cladosporium* (11.5%), and *Angustimassarina* (10.0%) (Table S1).

Heatmap visualization of the 40 most abundant genera highlighted substantial variation among individual trees while also revealing compartment-associated abundance patterns (Figure 5). Several genera, including *Diaporthe*, *Peniophora*, and *Talaromyces*, were consistently enriched in bark samples, whereas *Vishniacozyma*, *Sporobolomyces*, and *Alternaria* were more frequently associated with leaf and stem tissues.

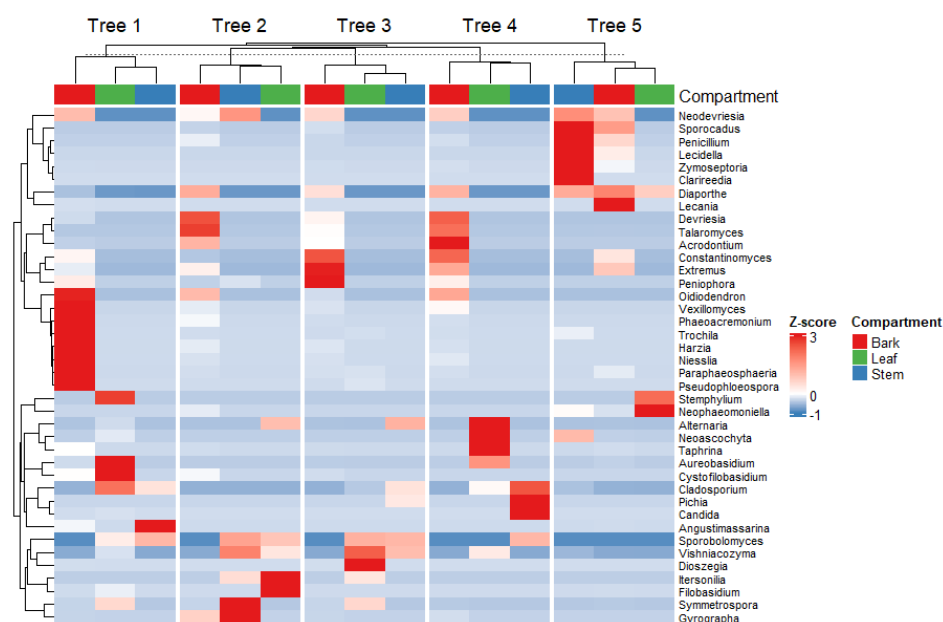


Figure 5. Heatmap of the 40 most abundant fungal genera detected across leaf, stem and bark compartments of five *Eucalyptus cinerea* trees. Relative abundances were Z-score normalised prior to visualization. Fungal genera were hierarchically clustered using Euclidean distances, while samples are displayed according to tree identity and compartment.

Differential abundance analysis identified 26 ASVs that differed significantly between bark and leaf, and 23 ASVs that differed significantly between bark and stem samples ($FDR < 0.05$, $baseMean > 10$) (Figure 6). No ASVs differed significantly between leaf and stem compartments. This pattern was consistent with the overlap observed between leaf and stem communities in both Bray–Curtis and weighted UniFrac ordinations. Several of the significantly enriched bark-associated ASVs were assigned to *Diaporthe*, *Peniophora*, *Neodevriesia*, *Niesslia*, and *Trochila*. Complete DESeq2 results, including taxonomic assignments, baseMean values, $\log_2$ fold-change, and adjusted p -values, are provided in Supplementary Table S2.

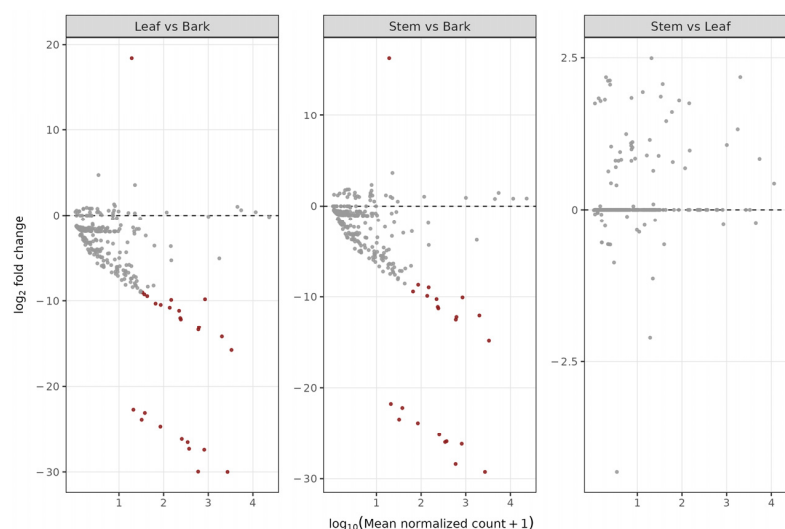


Figure 6. MA (mean-average) plot of differential fungal ASV abundance between compartments, as generated by DESeq2. The x-axis shows the mean normalised count (baseMean), and the y-axis shows the $\log_2$ fold-change relative to the reference compartment. Red points denote significant differences ($FDR < 0.05$) in ASVs.

Indicator species analysis identified 27 fungal genera and 28 fungal species significantly associated with bark samples (Tables S3 and S4). Representative bark-associated taxa included *Diaporthe*, *Peniophora*, *Cadophora*, *Paraphaeosphaeria neglecta*, *Niesslia tenuis*, and *Talaromyces clemensii*. Indicator values ranged from 0.84 to 1.00, with several taxa exhibiting perfect association with bark samples under the IndVal framework.

3.4. Phylogenetic Diversity

Phylogenetic diversity analyses were conducted to assess the evolutionary breadth of fungal communities. Faith's phylogenetic diversity (PD) differed significantly among bark, leaf and stem compartments (Kruskal–Wallis test, $H = 9.62$, $p = 0.008$), with bark samples showing substantially higher mean PD values than leaf or stem samples (Figure 7). Pairwise Mann–Whitney tests with Bonferroni correction indicated significant differences between bark and leaf (adjusted $p = 0.027$) and bark and stem (adjusted $p = 0.027$), but not between leaf and stem (adjusted $p = 1.0$).

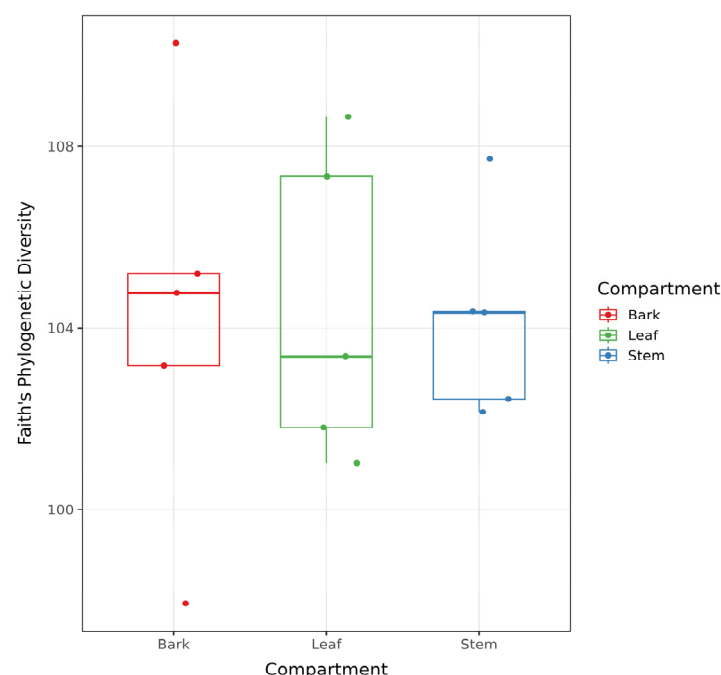


Figure 7. Faith's phylogenetic diversity (PD) of fungal communities associated with bark, leaf and stem compartments of *Eucalyptus cinerea*. PD values were calculated from fungal ASVs using a Grafen-transformed ITS phylogeny. Boxes represent the interquartile range, horizontal lines indicate medians, and points represent individual samples.

4. Discussion

This study presents a compartment-resolved assessment of fungal communities associated with the phyllosphere of intensively managed *Eucalyptus cinerea* grown for cut foliage production in Ireland. Collectively, the results indicate compartment-associated differences in fungal community composition, with bark supporting a distinct fungal assemblage compared to leaf and stem tissues. Differences among compartments were evident in taxonomic composition, differential abundance analyses, indicator taxa, and phylogeny-informed community structure, while leaf and stem communities remained broadly similar across multiple analytical approaches.

Observed fungal richness, Shannon and Simpson indices, and Faith's phylogenetic diversity were higher in bark than in leaf and stem tissues. These results suggest that bark samples exhibited greater taxonomic and phylogenetic diversity to photosynthetic tissues.

Similar compartment-associated differences in microbial diversity have been reported in plant microbiome studies and are often linked to variation in substrate characteristics, nutrient availability, tissue chemistry, and environmental exposure [8,37]. As a perennial woody tissue, bark represents a structurally stable and lignified habitat that differs markedly from leaves and young stems, and this long-lived, structurally complex substrate may accumulate a richer and more varied assemblage of fungal colonists than the comparatively short-lived surfaces of leaves and young stems.

Taxonomic analyses further supported the existence of compartment-specific fungal communities. Bark communities were strongly dominated by *Diaporthe*, with additional contributions from *Peniophora*, *Talaromyces*, and several taxa identified through indicator species analysis. In contrast, leaf and stem tissues were characterised by communities characterised by lower diversity and a more even distribution of dominant genera, including *Vishniacozyma*, *Sporobolomyces*, *Alternaria*, and *Cladosporium*. These patterns suggest that bark functions as a distinct above-ground microhabitat within the *E. cinerea* phyllosphere. Similar compartmentalisation of microbial communities has been observed in other woody plant species, where differences in physical tissue structure and chemical variation among tissues contribute to differences in microbial assemblages [9,10]. However, the ecological roles of the identified taxa were not investigated in the present study, and therefore ecological interpretations should be made cautiously.

Beta diversity analyses indicated that both compartment and tree identity significantly influenced fungal community composition. Tree identity explained a greater proportion of variation than compartment, suggesting that host-level characteristics or local environmental conditions may contribute substantially to fungal community assembly. The significant compartment effect observed in PERMANOVA, together with the differential abundance, indicator species, and taxonomic composition analyses, further supports the presence of compartment-associated variation in fungal communities. These findings suggest that fungal community structure is influenced by both tissue type and individual tree effects, highlighting the importance of considering multiple sources of variation when characterising plant-associated microbiomes [8].

Phylogeny-informed analyses provided additional insights into fungal community structure. Faith's PD was significantly higher in bark than in leaf and stem tissues, while weighted UniFrac ordination indicated separation of bark-associated communities from leaf and stem tissues. These results suggest that compartment-associated differences involved both taxonomic composition and distribution of phylogenetically distinct fungal lineages.

From an applied perspective, understanding fungal community structure in managed *Eucalyptus* systems is relevant for crop health, productivity, and resilience. Phyllosphere and endophytic fungi have been associated with plant stress responses, pathogen interactions, and host performance in a range of woody species [38,39]. The detection of taxa associated with saprotrophic, yeast-forming, and potentially latent pathogenic lifestyles suggests that *E. cinerea* supports a complex fungal assemblage under Irish production conditions. However, the ecological and functional significance of these communities remains unresolved and warrants further investigation.

The fungal taxa identified in this study likely represent a range of ecological lifestyles, including epiphytic, endophytic, saprotrophic, and potentially latent pathogenic associations. However, the ecological significance and functional roles of these taxa within *E. cinerea* were not assessed directly. As highlighted by Rodriguez et al., (2009), plant-associated fungal communities represent complex symbiotic systems that may influence host fitness, stress responses, and broader ecosystem functioning [39]. The coexistence of diverse fungal taxa within individual plants suggests that community-level interactions, rather than the activity of individual species alone, may contribute to plant performance.

Understanding these interactions will require approaches that move beyond descriptive community profiling towards functional and system-level investigations of plant–microbe associations [40].

Several limitations should be considered when interpreting these findings. Samples were collected from a single commercial site at one sampling time point, limiting the ability to assess temporal, seasonal or geographic variation in fungal communities associated with *E. cinerea*. In addition, the number of biological replicates per compartment was limited, and therefore statistical interpretations, particularly those relating to PERMANOVA analyses, should be treated with caution. Amplicon-based ITS sequencing provides limited functional resolution and may also be influenced by primer and amplification biases. Furthermore, pooled tissue sampling and the inability to distinguish between epiphytic and endophytic fungi may influence the observed community profiles. Future studies incorporating longitudinal sampling, environmental metadata, and functional approaches such as metagenomics, culturing, or transcriptomics would provide a more comprehensive understanding of fungal community dynamics in managed *Eucalyptus* systems.

5. Conclusions

Our results provide a baseline characterisation of fungal communities associated with the phyllosphere of intensively managed *Eucalyptus cinerea* grown for cut foliage production in Ireland. Fungal richness, alpha diversity, phylogenetic diversity, and taxonomic composition differed among leaf, stem and bark compartments, while host identity explained a substantial proportion of the observed variation in fungal community structure. Bark-associated communities displayed distinct phylogenetic and taxonomic patterns compared with photosynthetic tissues, suggesting compartment-associated variation within the *E. cinerea* phyllosphere.

The findings highlight the complexity of fungal assemblages associated with managed woody crops and reinforce the importance of integrating taxonomic, phylogenetic, and ecological perspectives in plant microbiome research. While this study provides an initial insight into fungal community structure in *E. cinerea*, further work incorporating larger sample sizes, multiple sites, and functional approaches will be required to better understand the ecological significance of these fungal communities and their potential roles in plant health, resilience, and crop productivity.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/applmicrobiol6070076/s1>. Figure S1. Percentage variance explained across principal coordinate axes derived from Bray–Curtis dissimilarities of fungal ASV abundances; Figure S2. NMDS Shepard plot showing goodness-of-fit of the non-metric multidimensional scaling ordination; Table S1. Relative abundance (%) of fungal taxa across bark, leaf and stem compartments of *Eucalyptus cinerea* at phylum, class, order, family, genus and species levels; Table S2. Significant differentially abundant fungal ASVs identified by DESeq between bark and leaf, and bark and stem compartments of *Eucalyptus cinerea* (FDR-adjusted $p < 0.05$; baseMean > 10); Table S3. Indicator genus analysis showing fungal genera significantly associated with bark, leaf or stem compartments based on the IndVal framework; Table S4. Indicator species analysis showing fungal species significantly associated with bark, leaf or stem compartments based on the IndVal framework.

Author Contributions: Conceptualization, T.B. and D.S.R.; methodology, T.B. and D.S.R.; data analysis, T.B.; writing, T.B. and D.S.R.; visualization, T.B.; project administration, D.S.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Teagasc internal funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw sequencing data generated in this study are publicly available through the National Centre for Biotechnology Information (NCBI) Sequence Read Archive under BioProject accession number PRJNA1460758. Bioinformatic workflows and analysis scripts are available on GitHub at: <https://github.com/ByrneTforestry/its-fungal-amplicon-Eucalyptus> (9 April 2026).

Acknowledgments: The authors would like to thank Teagasc for funding this work, Andy Welton (Teagasc, Vegetable & Cut Foliage Specialist Adviser) for help identifying the site, and the staff and owners at Tanners Foliage for permission to sample trees. Generative AI tools were used to assist with language editing. All scientific interpretation, data analysis, and final approval of the manuscript were performed by the authors.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ASV	Amplicon sequence variant
DADA2	Divisive Amplicon Denoising Algorithm 2
DESeq2	Differential expression analysis for sequence count data
FDR	False discovery rate
ITS	Internal transcribed spacer
MAFFT	Multiple alignment using fast Fourier transform
NMDS	Non-metric multidimensional scaling
PD	Faith's phylogenetic diversity
PCoA	Principal coordinates analysis
PE	Paired end
PERMANOVA	Permutational multivariate analysis of variance
QC	Quality control
Q-score	Quality score

References

1. Coppen, J.J. *Eucalyptus: The Genus Eucalyptus*; CRC Press: Boca Raton, FL, USA, 2002.
2. Bernstein, N.; Shushan, S.; Shargil, D.; Perzelan, Y.; Salim, S.; Zadka, T.; Riov, J.; Sacks, M.; Spiegel, E.; Tamari, Y.; et al. Seasonal variation in leaf damage symptoms, mineral contents, and postharvest attributes of cut foliage of *Eucalyptus* silver dollar (*E. cinerea*). *Isr. J. Plant Sci.* **2017**, *64*, 63–79. [\[CrossRef\]](#)
3. Teagasc. *Eucalyptus for Cut Foliage Factsheet 2/20*; Teagasc: Carlow, Ireland, 2020; p. 5.
4. Mercado-Blanco, J.; Abrantes, I.; Barra Caracciolo, A.; Bevivino, A.; Ciancio, A.; Grenni, P.; Hryniewicz, K.; Kredics, L.; Proença, D.N. Belowground Microbiota and the Health of Tree Crops. *Front. Microbiol.* **2018**, *9*, 1006. [\[CrossRef\]](#) [\[PubMed\]](#)
5. Arnold, A.E. Endophytic fungi: Hidden components of tropical community ecology. In *Tropical Forest Community Ecology*; Wiley: Hoboken, NJ, USA, 2008; pp. 178–188.
6. Kumar, V.; Jain, L.; Chaudhary, S.; Soni, R. Endophytes as potential plant growth promoters in forestry: Recent advances and perspectives. In *Microbes in Agri-Forestry Biotechnology*; CRC Press: Boca Raton, FL, USA, 2022; pp. 241–262.
7. Dinesh, K.; Sushma, K.; Jahagirdar, S.; Savani, A.K. Endophytes in the Management of Crop Diseases: Sustainable Development Goals in Agriculture. In *Endophytes*; Apple Academic Press: Waretown, NJ, USA, 2026; pp. 177–209.
8. Vorholt, J.A. Microbial life in the phyllosphere. *Nat. Rev. Microbiol.* **2012**, *10*, 828–840. [\[CrossRef\]](#) [\[PubMed\]](#)
9. Giese, M.; Stefani, E.; Larger, S.; Pindo, M.; Farneti, B.; Ajelli, M.; Cattani, M.; Delgado-Baquerizo, M.; Coleine, C.; Donati, C. Highly compartmentalized microbiomes in blueberry microhabitats. *Front. Microbiol.* **2025**, *16*, 1732372. [\[PubMed\]](#)
10. Kranjec Orlović, J.; Diminić, D.; Ištók, I.; Volenec, I.; Hodak, L.; Grubešić, M.; Tomljanović, K. Fungal Presence and Changes of Wood Structure in Bark Stripping Wounds Made by Red Deer (*Cervus elaphus* L.) on Stems of *Fraxinus angustifolia* (Vahl). *Forests* **2024**, *15*, 314. [\[CrossRef\]](#)
11. Toju, H.; Tanabe, A.S.; Yamamoto, S.; Sato, H. High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PLoS ONE* **2012**, *7*, e40863. [\[CrossRef\]](#) [\[PubMed\]](#) [\[PubMed Central\]](#)
12. Chen, S.; Zhou, Y.; Chen, Y.; Gu, J. fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **2018**, *34*, i884–i890. [\[CrossRef\]](#) [\[PubMed\]](#)

13. Estaki, M.; Jiang, L.; Bokulich, N.A.; McDonald, D.; González, A.; Kosciulek, T.; Martino, C.; Zhu, Q.; Birmingham, A.; Vázquez-Baeza, Y.; et al. QIIME 2 Enables Comprehensive End-to-End Analysis of Diverse Microbiome Data and Comparative Studies with Publicly Available Data. *Curr. Protoc. Bioinform.* **2020**, *70*, e100. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Abarenkov, K.; Nilsson, R.H.; Larsson, K.-H.; Taylor, A.F.S.; May, T.W.; Frøslev, T.G.; Pawlowska, J.; Lindahl, B.; Pöldmaa, K.; Truong, C.; et al. The UNITE database for molecular identification and taxonomic communication of fungi and other eukaryotes: Sequences, taxa and classifications reconsidered. *Nucleic Acids Res.* **2024**, *52*, D791–D797. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Katoh, K.; Misawa, K.; Kuma, K.; Miyata, T. MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* **2002**, *30*, 3059–3066. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Price, M.N.; Dehal, P.S.; Arkin, A.P. FastTree: Computing Large Minimum Evolution Trees with Profiles instead of a Distance Matrix. *Mol. Biol. Evol.* **2009**, *26*, 1641–1650. [\[CrossRef\]](#)
18. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2025.
19. McMurdie, P.J.; Holmes, S. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE* **2013**, *8*, e61217. [\[CrossRef\]](#) [\[PubMed\]](#)
20. Valero-Mora, P.M. ggplot2: Elegant Graphics for Data Analysis. *J. Stat. Softw. Book Rev.* **2010**, *35*, 1–3. [\[CrossRef\]](#)
21. Dixon, P. VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* **2003**, *14*, 927–930. [\[CrossRef\]](#)
22. Magurran, A.E. *Measuring Biological Diversity*; Blackwell Publishing: Oxford, UK, 2004.
23. Chao, A. Nonparametric Estimation of the Number of Classes in a Population. *Scand. J. Stat.* **1984**, *11*, 265–270.
24. Chao, A.; Lee, S.-M. Estimating the Number of Classes via Sample Coverage. *J. Am. Stat. Assoc.* **1992**, *87*, 210–217. [\[CrossRef\]](#)
25. Shannon, C.E. A Mathematical Theory of Communication. *Bell Syst. Tech. J.* **1948**, *27*, 379–423. [\[CrossRef\]](#)
26. Simpson, E.H. Measurement of Diversity. *Nature* **1949**, *163*, 688. [\[CrossRef\]](#)
27. Dunn, O.J. Multiple Comparisons Using Rank Sums. *Technometrics* **1964**, *6*, 241–252. [\[CrossRef\]](#)
28. Bray, J.R.; Curtis, J.T. An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecol. Monogr.* **1957**, *27*, 326–349. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Gower, J.C. Some Distance Properties of Latent Root and Vector Methods Used in Multivariate Analysis. *Biometrika* **1966**, *53*, 325–338. [\[CrossRef\]](#)
30. Kruskal, J.B. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika* **1964**, *29*, 1–27. [\[CrossRef\]](#)
31. Jaccard, P. Etude de la distribution florale dans une portion des Alpes et du Jura. *Bull. Soc. Vaudoise Sci. Nat.* **1901**, *37*, 547–579. [\[CrossRef\]](#)
32. Anderson, M.J. A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* **2001**, *26*, 32–46. [\[CrossRef\]](#)
33. Chao, A.; Chiu, C.-H.; Jost, L. Phylogenetic Diversity Measures and Their Decomposition: A Framework Based on Hill Numbers. In *Biodiversity Conservation and Phylogenetic Systematics: Preserving Our Evolutionary Heritage in an Extinction Crisis*; Pellens, R., Grandcolas, P., Eds.; Springer International Publishing: Cham, Switzerland, 2016; pp. 141–172.
34. Lozupone, C.; Knight, R. UniFrac: A New Phylogenetic Method for Comparing Microbial Communities. *Appl. Environ. Microbiol.* **2005**, *71*, 8228–8235. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Kolde, R. pheatmap: Pretty Heatmaps. R Package Version 1.0.13. 2025. Available online: <https://github.com/raivokolde/pheatmap> (accessed on 9 April 2026).
36. Anders, S.; Huber, W. Differential expression analysis for sequence count data. *Genome Biol.* **2010**, *11*, R106. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Lindow, S.E.; Brandl, M.T. Microbiology of the Phyllosphere. *Appl. Environ. Microbiol.* **2003**, *69*, 1875–1883. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Arnold, A.E.; Lutzoni, F. Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? *Ecology* **2007**, *88*, 541–549. [\[CrossRef\]](#) [\[PubMed\]](#)
39. Rodriguez, R.J.; White, J.F., Jr.; Arnold, A.E.; Redman, R.S. Fungal endophytes: Diversity and functional roles. *New Phytol.* **2009**, *182*, 314–330. [\[CrossRef\]](#) [\[PubMed\]](#)
40. Jain, A.; Sarsaiya, S.; Singh, R.; Gong, Q.; Wu, Q.; Shi, J. Omics approaches in understanding the benefits of plant-microbe interactions. *Front. Microbiol.* **2024**, *15*, 1391059. [\[CrossRef\]](#) [\[PubMed\]](#)

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.