Lettuce seedlings rapidly assemble their microbiome from the environment through deterministic processes

Nesma Zakaria Mohamed [a,b], Leonardo Schena [a,**], Antonino Malacrinò [a,*]

[a] *Department of Agriculture, Università Degli Studi Mediterranea di Reggio Calabria, Reggio Calabria, Italy*
[b] *Agronomy Department, Faculty of Agriculture, Cairo University, Egypt*

ABSTRACT

Plant-associated microorganisms have significant impacts on plant biology, ecology, and evolution. Although several studies have examined the factors driving variations in plant microbiomes, the mechanisms underlying the assembly of the plant microbiome are still poorly understood. In this study, we used gnotobiotic plants to test (i) whether seedlings create a selective environment and drive the assembly of root and leaf microbiomes through deterministic or stochastic processes, and (ii) whether seedlings structure the microbiome that is transferred through seeds using deterministic processes and whether this pattern changes when seedlings are exposed to the environmental microbiome. Our results show that the microbiome of gnotobiotic plants (i.e., inherited through seeds) is not under the selective influence of the host plant but changes quickly when plants are exposed to soil microbiomes. Within one week, plants were able to select microorganisms from the inocula, assemble the root microbiome, and assemble the shoot microbiome. This study supports the hypothesis that plants at early developmental stages might exert strong selective activity on their microbiomes and contribute to clarifying the mechanisms of plant microbiome assembly.

Introduction

Plants grow in close association with a large and diverse community of microorganisms (e.g., bacteria, fungi, nematodes, and viruses) that have profound effects on plant biology, ecology, and evolution (Trivedi et al., 2020). Indeed, the plant microbiome can influence a multitude of host traits, including fitness, nutrient/water uptake, and resistance to biotic and abiotic stressors (Malacrinò et al., 2022; Song et al., 2020; Trivedi et al., 2020). The structure of the plant microbiome is highly variable throughout developmental stages, across and within plant organs, and between species and genotypes, and is influenced by the physiological status of the plant (Trivedi et al., 2020). While several studies have focused on describing the variation in microbiomes between different plants (e.g., between genotypes), within the same plant (e.g., between organs), or on inferring the effects of different factors (e.g., water, herbivory, pathogens, and agricultural practices) on the structure of the plant microbiome, little is known about the processes that drive the assembly of the plant microbiome. Understanding the mechanisms behind plant microbiome assembly is crucial for leveraging the power of plant-microbe interactions for sustainable agriculture

(Mittelstrass et al., 2021).

Plants acquire their microbiome either horizontally from the environment (e.g., soil and air) or vertically from seeds (Abdelfattah et al., 2019; Berg et al., 2021; Shade et al., 2017). Previous research has shown that soil is a major source of the plant microbiome, whereas the inherited microbiome has a smaller influence (Shade et al., 2017; Trivedi et al., 2020). Deterministic and stochastic processes are the major forces driving the assembly of plant microbiomes (Dini-Andreote et al., 2015). Deterministic processes (i.e., selection) influence the presence/absence and abundance of microbial taxa, and are driven by selective forces generated by the host plant or abiotic environment. Deterministic processes can generate dissimilar (variable selection) or similar (homogeneous selection) microbial communities. On the other hand, stochasticity (e.g., dispersal and drift) dominates when selection is weak, and non-selective processes are mainly responsible for driving the assembly of the plant microbiome, such as movements between communities (dispersal) and changes in population size due to random events (drift). Previous studies have focused on testing whether deterministic or stochastic processes dominate the assembly of the plant microbiome; however, results from previous studies have produced

---

contrasting outcomes. There is evidence of deterministic processes driving the assembly of leaf and root microbiomes (Cai et al., 2020; Dove et al., 2021; Francis et al., 2023; Guo et al., 2021; Moroenyane et al., 2021; Xiong et al., 2021; Yin et al., 2023), but also evidence suggesting that stochastic processes are dominant (Bell et al., 2022; Chen et al., 2022; Fu et al., 2023; Gao et al., 2020; Louisson et al., 2023; Wang et al., 2022). Some studies suggest that the assembly of bacterial and fungal communities is often driven by contrasting processes, which may vary according to the plant organ (Cai et al., 2020; Wang et al., 2023; Xiong et al., 2021; Yan et al., 2022). Other studies have suggested that the dominance of either deterministic or stochastic processes varies over time (Maignien et al., 2014; Xiong et al., 2021) or as an effect of stress (Gao et al., 2020; Kuang et al., 2023). Thus, current evidence does not show clear patterns in the processes driving the plant microbiome across different plant species.

Previous research has mainly focused on plants grown under field conditions that are already in an advanced stage of growth. This might not provide a complete picture of the dynamics behind the processes that drive plant microbiome assembly. For example, the contribution of deterministic and stochastic factors might change with plant development (Dini-Andreote and Raaijmakers, 2018; Maignien et al., 2014; Xiong et al., 2021) or plants at a later growth stage might exert a lower level of selection on their microbiome and direct resources to other tasks. Little is known about the processes driving microbiome assembly in plants during the early growth stages. Previously, it was suggested that the assembly of seedling-associated microbial communities might be highly subjected to priority effects and thus are mainly shaped by stochastic processes (Dini-Andreote and Raaijmakers, 2018). However, a few studies have shown that the plant microbiome at the early stages is assembled in a selective environment (Wang et al., 2023; Xiong et al., 2021), which might be driven by plants (Rochefort et al., 2021). Indeed, creating a selective environment and directing microbiome assembly processes might be more important for plants at early stages of growth, as this might help them gather beneficial microorganisms to aid plant nutrition and protection against pathogens.

In this study, we used gnotobiotic plants to gain further understanding of the processes driving the assembly of plant microbiomes, particularly immediately after germination, as this is a crucial step when plant-microbiome interactions are established. Lettuce plants (*Lactuca sativa* L.) were grown under gnotobiotic conditions (in this case, germinated from surface-sterilized seeds and transferred to a sterile system) and exposed to 21 different soil microbial communities. After one week, we collected samples (roots and shoots) for amplicon metagenomic (16S and ITS) analyses. First, we investigated our hypothesis that seedlings create a selective environment belowground and, through selection, drive the assembly of root and leaf microbiomes through deterministic processes. Second, we tested whether seedlings exert the same selective forces on the microbiome that is transferred through seeds, or whether selective forces come into play when seedlings are exposed to the environmental microbiome. We hypothesized that the inherited seed microbiome is not subjected to further selective forces (Shade et al., 2017), but selection occurs quickly once plants are exposed to complex soil microbial communities.

Methods

Experimental procedure

Lettuce seeds (variety "Romabella") were surface-sterilized using the method of Davoudpour et al. (2020) with a few modifications. Briefly, lettuce seeds were treated with 70% ethanol for 3 min before being sterilized twice with a 30 mL mixture of 8% sodium hypochlorite and 17 μL Tween 20 (15 min each round, 30 min in total). The seeds were thoroughly rinsed five times with sterile water. Sterilized seeds were then placed on wet filter paper in a sterile Petri dish for approximately ten days under direct sunlight for germination. Seed sterility was

checked by placing 15 seeds on PDA medium and incubating them for approximately 5 days at 20 °C, after which no microbial growth was observed. Surface sterilization was performed to remove external contaminants that could rapidly grow in sterilized soil and influence plant growth.

The experiment was performed using sterile microboxes (Combiness Europe, Nevele, Belgium; 14 cm H × 9 cm base ∅, 1 L volume) commonly used for micropropagation, allowing plant growth under sterile conditions. Each microbox was sterilized for 10 min with 4% sodium hypochlorite before being autoclaved at 121 °C for 15 min, filled with approximately 170 g of autoclaved soil, and then watered with 10 mL of autoclaved water. The soil was prepared by sieving the soil to 1 mm to remove large particles, which were watered, covered, and left for approximately 7 day at ~20 °C to allow the growth and development of microorganisms. After that, the soil was autoclaved at 121 °C for 3 h, allowed to cool to room temperature for approximately 24 h, and autoclaved again at 121 °C for 3 h before being used to fill the sterile microboxes.

Five seedlings (~1 week old) were transplanted into each microbox and inoculated with 1 mL of different soil inocula (see below). Each microbox was inoculated with a different inoculum ($n = 21$). In addition, four microboxes were inoculated with 1 mL of distilled water as a control. Soil inocula and sterile water were added to the soil to avoid direct contact with seedlings. After that, all boxes were kept under direct sunlight at room temperature (24–25 °C) and rearranged every 24 h to account for variation in light exposure. Seven days after soil inoculation, all the plants in each microbox were gently collected using their entire root system. Plants were rinsed with autoclaved water to remove soil particles before being dissected into two parts (shoots and roots) and placed separately in 2 mL Eppendorf tubes, pooling all five seedlings from the same microbox. Subsequently, all samples were freeze-dried for 24 h and then crushed for 1 min at 30 Hz using a bead mill homogenizer and 2–3 glass beads (3 mm ∅). Finally, the samples were stored at −80 °C until DNA extraction.

Soil inocula

Soils were collected from different areas with varying cultivation methods, crops, forests, and uncultivated land to obtain a diverse microbial community between each inoculum. Each soil inoculum was prepared according to the method described by Walsh et al. (2021) with minor modifications. Briefly, 20 g of soil was transferred to a 50 mL sterile falcon tube filled with 20 mL of sterile distilled water. The tubes were then vortexed for approximately 30 s before being centrifuged at 1000×g for 1 min to sediment the larger soil particles. The supernatant was transferred to a new Falcon tube and the microbes were pelleted by centrifugation at 4000×g for 5 min. The supernatant was discarded, and the pellets were resuspended in 20 mL of sterile phosphate-buffered saline (PBS). A small aliquot of each inoculum was stored at −80 °C for further processing.

DNA extraction, amplicon library preparation and sequencing

DNA from the roots and shoots was extracted using the PowerPlant® Pro DNA Isolation Kit (MO BIO), whereas DNA from the inocula was extracted using the PowerSoil® DNA Isolation Kit (MO BIO), according to the manufacturer's instructions. The DNA concentration and quality were estimated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Bacterial communities were characterized by amplifying a portion of the 16S rRNA gene using the primers 515f/806r (Caporaso et al., 2012), while fungal communities were characterized by amplifying the ITS2 region using the primer pair ITS3-KYO2/ITS4 (Toju et al., 2012). Amplifications were performed using standard two-step PCR, first amplifying the target fragment and then ligating the adaptors/barcodes for sequencing (see for example our previous study Malacrinò et al., 2021). Amplifications included: (i) non-template controls

($n = 3$), where DNA extraction was performed by replacing samples with nuclease-free water to account for possible contamination of instruments, reagents, and consumables used for DNA extraction; and (ii) negative PCR controls ($n = 3$), in which the DNA template for PCR was replaced with the same volume of ultrapure water. Libraries were then quantified using a Qubit fluorometer (Thermo Fisher Scientific), pooled together at equimolar ratios, and sequenced on an Illumina MiSeq platform (Illumina, CA, USA) using the MiSeq Reagent Kit v3 600 cycles (300 PE) following the supplier's instructions.

Data analysis

Data analysis was performed using R v4.1.2 (R Core Team, 2020) and visualizations were created using *ggplot2* (Wickham, 2009). Paired-end reads were processed using the *DADA2* pipeline (Callahan et al., 2016) to remove low-quality data, identify Amplicon Sequence Variants (ASVs) and remove chimeras. Taxonomy was assigned using SILVA v138 (Quast et al., 2013) for bacteria, and UNITE v8.2 (Nilsson et al., 2019) for fungi. The ASV table, metadata, taxonomical annotation for each ASV, and phylogenetic tree of all ASVs were merged into a *phyloseq* object (McMurdie and Holmes, 2013) for handling. Before downstream analyses, all ASVs identified as "chloroplast" or "mitochondria" were discarded, and the package *decontam* (Davis et al., 2018) was used to remove potential contaminants using data from non-template and negative controls (see above). ASV sequences were aligned using *DECIPHER* (Wright, 2015) and bootstrapped maximum-likelihood phylogenetic trees were estimated using phangorn (Schliep, 2011). After removing singletons, the ASV table was normalized using the package *wrench* (Kumar et al., 2018) and used for all analyses, except when calculating the diversity metrics (observed richness and Faith's, Shannon's, and Simpson's indices), which were integer numbers that needed to be used. All analyses were performed separately for bacterial and fungal communities.

Differences in the multivariate structure of microbial communities between the three compartments (inocula, roots, and shoots) were tested using PERMANOVA (999 permutations) on both a weighted UniFrac and an unweighted UniFrac distance matrix between samples, performed using the package *vegan* (Dixon, 2003). Results were visualized using a NMDS (non-metric Multi-Dimensional scaling) procedure, and pairwise contrasts were inferred using the package *RVAideMemoire* (Hervé, 2022) correcting p-values using the FDR (False Discovery Rate (FDR) procedure.

The diversity of microbial communities within each sample was estimated using Faith's phylogenetic diversity index calculated using the package *picante* (Kembel et al., 2010) and the observed richness, Shannon's diversity, and Simpson's dominance indices were calculated using the package *microbiome* (Sudarshan and Shetty, 2017). Differences between compartments (inocula, roots, shoot) were tested using the packages *lme4* (Bates et al., 2015) and *car* (Fox and Weisberg, 2018) by fitting a separate linear model for each diversity index and using "compartment" as fixed factor. Pairwise contrasts were inferred using the package *emmeans* (Lenth, 2022), correcting p-values using the False Discovery Rate (FDR) procedure.

ASVs that were differentially abundant between pairs of compartments (inocula, roots, and shoots) were identified using the package *MaAsLin2* (Mallick et al., 2021), using an adjusted p-value of 0.05. We also calculated the number of ASVs shared between pairs of compartments and all three compartments for each inoculum, and repeated this procedure by randomizing the values within the ASV table. We then tested for differences between the two distributions (observed vs. random number of shared ASVs between compartments) using a generalized linear mixed-effects model, with category (observed, random) as a fixed effect and the inoculum ID as a random variable.

The beta Nearest Taxon Index (βNTI, quantifying the deviation of Mean Nearest Taxon Distances from null expectations) was calculated using the package *picante* and used to test whether microbial communities assembled following deterministic or stochastic assembly processes (Arnault et al., 2022; Larsen et al., 2023). The RCbray index was estimated using the package *iCAMP* (Ning et al., 2020).

Results

Amplicon metagenomic sequencing yielded 9,291,816 raw reads for 16S rRNA and 6,904,536 raw reads for ITS. After cleanup and removal of plastidial reads, the 16S dataset included 964,167 reads (average 14,608 reads/sample; min 1,009; max 41,018; Fig. S1A, Fig. S2), whereas the ITS dataset included 1,570,286 reads (average 26,171 reads/sample; min 1,244; max 76,034; Fig. S1B, Fig. S3).

First, we tested whether the plant microbiome (roots and shoots) differed from the composition of the inoculated microbial communities. We found that the multivariate structure of microbial communities differed among inocula, shoots, and roots (Fig. 1) using both a weighted (bacteria $F_{1, 63} = 12.5$, $R^2 = 0.28$, $p < 0.001$; fungi $F_{1, 57} = 11.73$, $R^2 = 0.29$, $p < 0.001$) and an unweighted UniFrac distance matrix (bacteria $F_{1, 63} = 7.75$, $R^2 = 0.19$, $p < 0.001$; fungi $F_{1, 57} = 7.03$, $R^2 = 0.21$, $p < 0.001$). For bacterial and fungal communities, post-hoc contrasts showed differences between the structures of microbiomes in the inocula, shoots, and roots ($p = 0.001$ for all pairwise comparisons, FDR-corrected). Post-hoc tests highlighted a difference in the multivariate structure between root and shoot bacterial communities (UniFrac, $p = 0.04$; weighted UniFrac, $p = 0.01$; FDR-corrected) but not for fungal communities (UniFrac $p = 0.81$, weighted UniFrac $p = 0.68$, FDR-corrected).

When focusing on the diversity of bacterial microbial communities, we found differences in phylogenetic diversity ($F_{2, 63} = 282.21$, $p < 0.0001$, Fig. 2A), Shannon's diversity index ($F_{2, 63} = 27.42$, $p < 0.0001$, Fig. 2B), Simpson's diversity index ($F_{2, 63} = 9.16$, $p = 0.0003$, Fig. 2C), and observed richness ($F_{2, 63} = 33.76$, $p < 0.0001$, Fig. 2D). Post-hoc contrasts showed higher phylogenetic diversity (Fig. 2A), Shannon diversity (Fig. 2B), and observed richness (Fig. 2D) in the inocula and roots than in the shoots, and a higher dominance in the shoots than in the other two compartments (Fig. 2C). Similarly, in the fungal community, we also found differences in phylogenetic diversity ($F_{2, 57} = 505.41$, $p < 0.0001$, Fig. 2E), Shannon's diversity index ($F_{2, 57} = 280.79$, $p < 0.0001$, Fig. 2F), Simpson's diversity index ($F_{2, 57} = 38.97$, $p < 0.0001$, Fig. 2G), and observed richness ($F_{2, 63} = 498.44$, $p < 0.0001$, Fig. 2H). Both roots and shoots showed lower microbial diversity and richness (Fig. 2E, F, H) and higher dominance (Fig. 2G) than the inocula, but there was no difference between the two plant compartments (Fig. 2E–H).

To test the hypothesis that the root microbiome is assembled from the inoculum and that the shoot microbiome is further selected from the root microbiome, we identified changes in ASV abundance between pairs of compartments. We found that 336 bacterial ASVs were enriched in the roots compared to the inocula, and 137 ASVs were enriched in the shoots compared to the inoculum (Fig. 3A and B). Although 87 bacterial ASVs were enriched in both roots and shoots compared to the inocula, no ASV was significantly enriched in the shoots compared to the roots (Fig. 3C). In contrast, only one fungal ASV was significantly enriched in the roots compared to the inoculum (Fig. 3D), and no ASV was enriched in the shoots compared to the inoculum (Fig. 3E) or roots (Fig. 3F).

Further tests showed that the number of ASVs shared between compartments (Fig. 4A and B) was always different from random chance, except for fungal ASVs that were shared between all compartments (Fig. 4B). While the number of ASVs shared between compartments was lower than random chance in most cases, the number of bacterial ASVs shared between roots and shoots was higher than random (Fig. 4A). In addition, the number of bacterial and fungal ASVs unique to shoots was always lower than that of simulated random microbial communities, whereas the number of ASVs unique to the inoculum was always higher than random chance (Fig. 4).

To further explore the idea that the microbiome of gnotobiotic plants is assembled through deterministic processes rather than stochastic
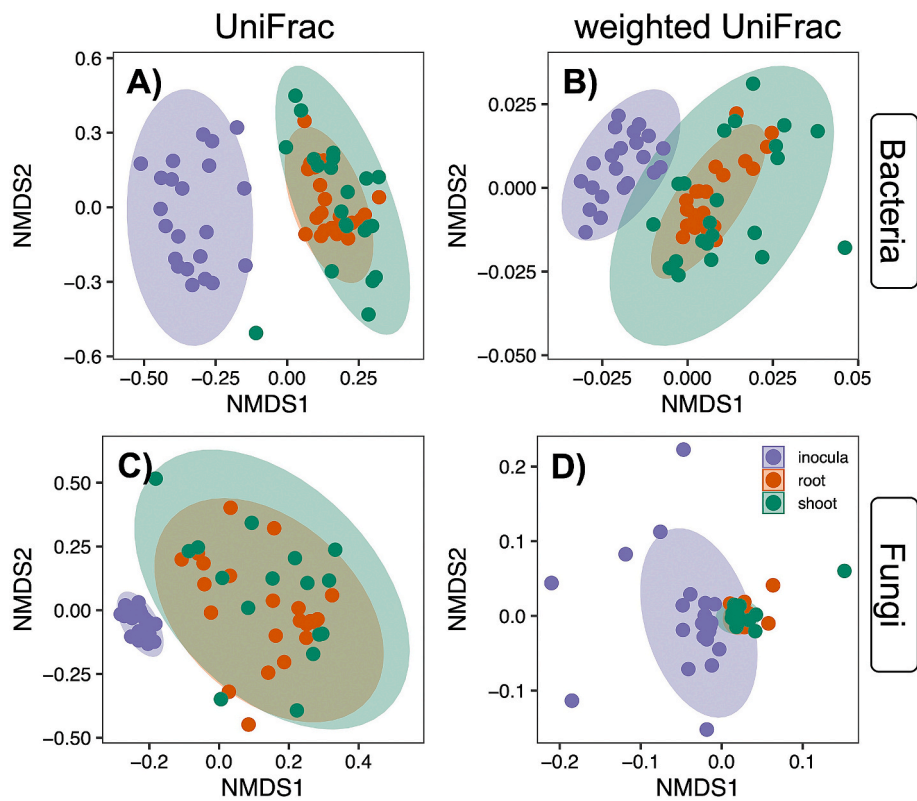
Fig. 1. NMDS (Non-Metric Multi Dimensional Scaling) of bacterial (A, B) and fungal (C, D) communities of inocula, root, and shoot samples, using unweighted (A, C) and weighted (B, D) distances between samples. Inocula were collected at the experimental setup, shoot and root samples were collected after 7 days from experimental setup. Ellipses represent the 95% CI for each compartment.
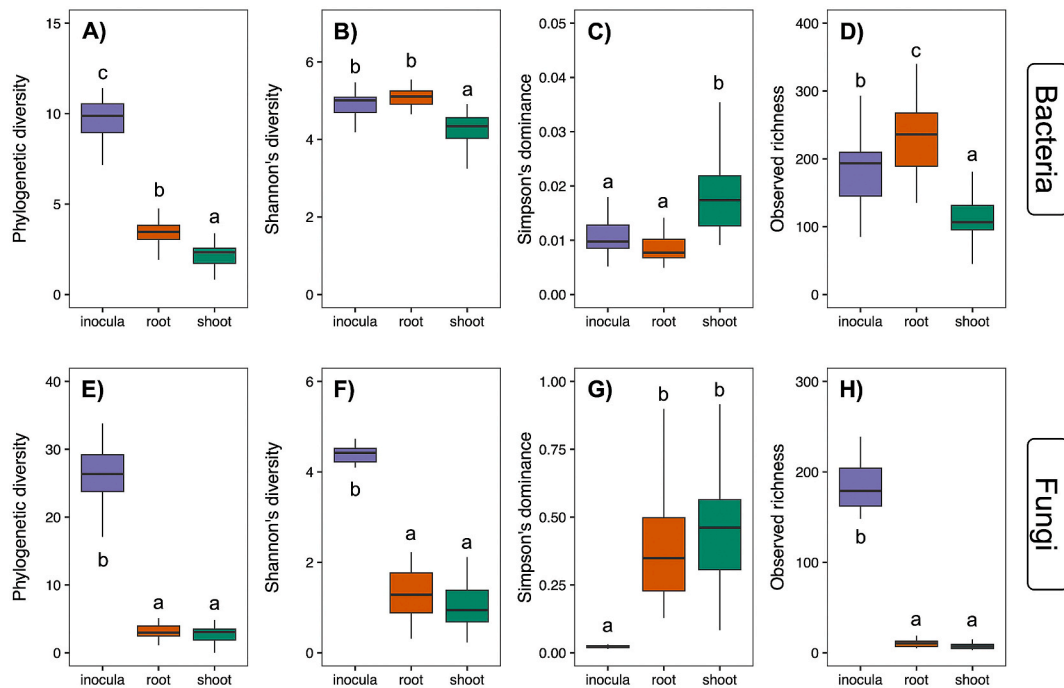

Fig. 2. Phylogenetic diversity (A and E), Shannon diversity (B and F), Simpson dominance (C and G), and observed richness (D and H) indexes for bacterial (A, B, C, D) and fungal (E, F, G, H) communities in samples collected from inocula, root, and shoot. Inocula were collected at the experimental setup, shoot and root samples were collected after 7 days from experimental setup. Pairwise comparisons are shown as letters for each boxplot, and exact p-values are reported in Table S1.
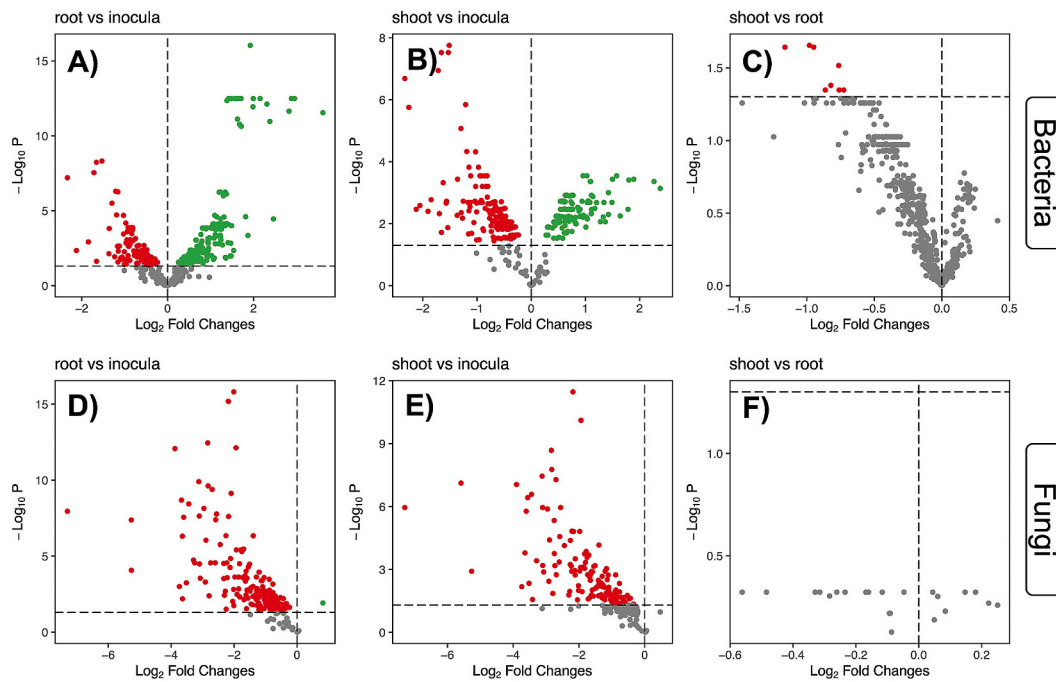
Fig. 3. Volcano plots showing differentially abundant ASVs of bacteria (top) and fungi (bottom) between pairs of compartments. (A and D) root (green) vs inocula (red), (B and E) shoot (green) vs inocula (red), (C and F) shoot (green) vs roots (red). ASVs in grey are not differentially abundant between the two compartments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
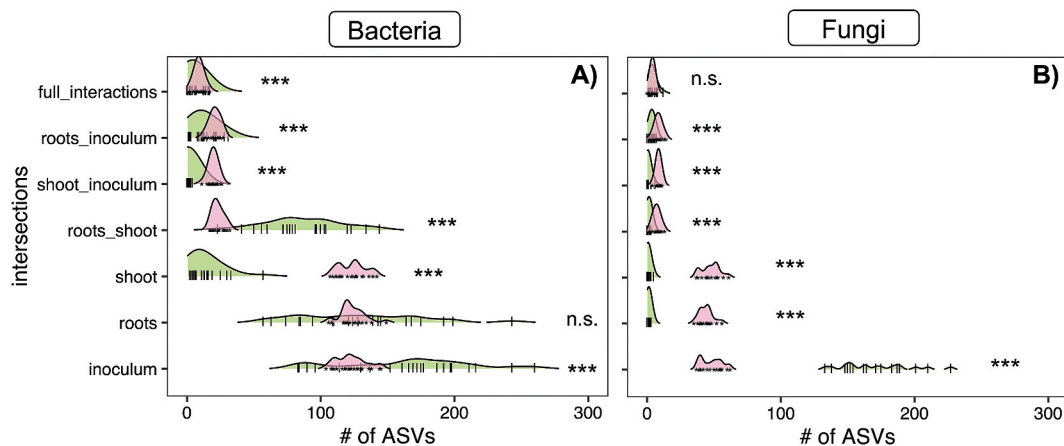

Fig. 4. Ridgeline plot showing the distribution of the number of ASVs shared between compartments, and testing the difference between observed data (green distribution, vertical lines are individual datapoints) and randomized data (pink distribution, small stars are individual datapoints). Asterisks on the side of each pair of ridgelines show the results from a *lmer* testing for differences between the means of the two distributions (***p < 0.001, *n. s.* P > 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

associations from the inoculated microbial communities, we tested the assembly processes of the microbial communities associated with plants that were not inoculated, thus interacting only with microbes that were inherited from the seeds. Thus, when examining the composition of the microbial communities associated with plants that had not been inoculated, we observed a community composed of 172 bacterial and 9 fungal ASVs (Fig. S4). When examining the composition of these communities, we found that the composition was highly variable between samples, suggesting that microbiome assembly under gnotobiotic conditions followed stochastic rules. We further tested this idea by calculating the βNTI index for both bacterial and fungal communities and found that the βNTI of both root and shoot microbiomes was between −2 and 2 (Fig. 5A and B), suggesting that stochastic processes are the major driver of microbiome assembly in plants associated with

microorganisms derived solely from seeds. In addition, the RCbray index for roots was on average 0.35 (bacteria) and 0.04 (fungi), while for shoot was on average 0.28 (bacteria) and −0.05 (fungi). We then examined plants that had been inoculated with microorganisms from the field and found that the βNTI index was >2 for both bacteria and fungi (Fig. 5C and D), suggesting that deterministic processes contributed to the assembly of plant microbiomes.

Discussion

In this study, we showed that gnotobiotic plants can quickly select inoculated microbial communities and assemble root and shoot microbial communities via deterministic processes. First, our results showed that the structure of the microbial communities associated with the
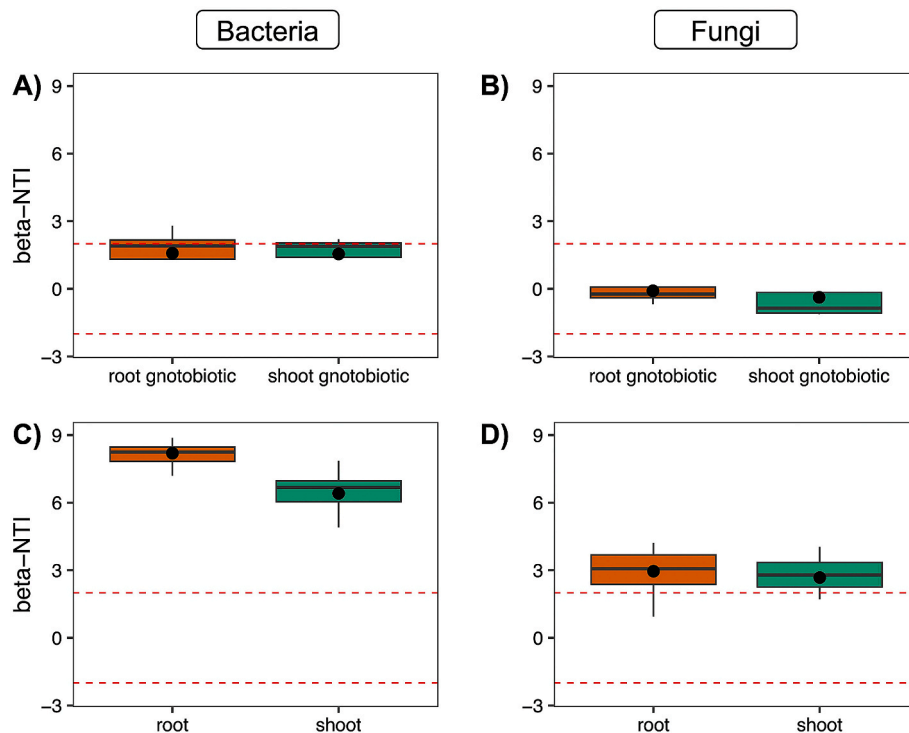
Fig. 5. βNTI (beta-nearest taxon index) of bacterial (A and C) and fungal (B and D) communities associated with gnotobiotic (A and B) and inoculated (C and D) lettuce plants. Horizontal dashed lines represent reference values of −2 and 2, indicating that the thresholds in the microbiome assembly are considered to be shaped by stochastic processes. The black dot within each boxplot represents the distribution mean.

inoculated plants was different from that of the inoculum, and that the bacterial diversity was higher in the roots than in the shoots. This result suggests that the plants created a selective environment in the root and shoot compartments. We observed a high proportion of ASVs with differences in abundance between roots/shoots and the inoculum, whereas no ASV was significantly enriched in the shoot compared to the roots. This suggests that plants exert a selective force on root-associated microbial communities, and from this, they are able to assemble the shoot microbiome. Finally, we directly tested the prevalence of deterministic or stochastic assembly processes in both the gnotobiotic and inoculated plants. Our results suggest that microbial communities in gnotobiotic plants, which are built from microbial taxa inherited from seeds, are not driven by selection processes but by ecological drift (RCbray index <0.95) (Ning et al., 2020). However, once inoculated, plants were able to quickly (one week) assemble root and shoot microbiomes through deterministic processes. Taken together, our results support our hypothesis that seedlings create a selective environment belowground and that through selection from the soil microbial community, they assemble both root and shoot microbiomes. In addition, we found support for our hypothesis that the inherited seed microbiome is subjected to stochastic assembly processes, whereas, once exposed to the environmental microbiome (i.e., inocula in our case), plants can exert selective pressure and assemble their microbial communities through deterministic processes.

When testing the assembly processes of bacterial and fungal communities in maize under field conditions and across developmental stages, Xiong et al. (2021) found that plant bacterial communities were assembled through selection at early growth stages. Similar results were observed in the wetlands of *Typha orientalis* (Wang et al., 2023), where the microbiome of seedlings showed signatures of selection rather than stochasticity in their assembly. In both cases, samples were collected from the field, and while this ensured that the results hold in real-life conditions, these observations might be biased by external factors that might influence the assembly processes of the plant microbiome. In the present study, we used a reductionist approach to grow gnotobiotic

plants and exposed them to a range of inocula, thus removing possible interference from the air microbiome and abiotic effects. Indeed, all the handling procedures were performed under a microbiological hood, and microboxes are built with a filter that prevents microorganisms from the surrounding environment to enter the microbox, while allowing gas exchanges. Thus, we were able to distinguish the selection effect driven by the plant from other possible factors driving selection on the soil microbiome. Most previous studies have taken a snapshot of a particular stage of plant development, while Xiong et al. (2021) followed maize plants throughout development and found that the assembly of plant bacterial communities was dominated by deterministic processes early in development, while stochastic processes were more dominant later in the growth season. This matches our results in that plants at early stages exert a strong selection on their microbiome. While more data needs to be gathered, this might support the idea that plants vary the strength of the selection they impose on their microbiome across development, in the same way they redirect resources (e.g., root exudates) at more mature developmental stages (Badri and Vivanco, 2009).

Several other studies have focused on understanding the ecological processes driving the assembly of the plant microbiome, and as reported in the introduction, the results vary greatly across plant phylogeny, geography, and plant organs. This variation may be caused by several factors (e.g., plant genotype and stressors) that are known to influence the plant microbiome (Trivedi et al., 2020) and are difficult to account for in field settings. For example, differences in the strength of selection have been observed across rice genotypes (Yin et al., 2023) or in response to biotic and abiotic stressors (Gao et al., 2020; Kuang et al., 2023). However, these patterns appear to be conserved among closely related species. Wang et al. (2022) and Yan et al. (2022) found that stochastic processes drive the assembly of microbial communities associated with *Eucalyptus* plants. Similarly, Guo et al. (2021) and Yin et al. (2023) observed deterministic processes that guided the assembly of rice microbiomes. Our study was performed under heavily controlled conditions; thus, it would be useful to disentangle the plant-driven effect from other factors that might bias the results. However, this information

needs to be paired with experiments under field conditions, where other factors might mask the microbiome selection driven by the host plant. This is a key step towards disentangling the effects of the host plant within the holobiont.

Our results also show that the deterministic processes driving the assembly of shoot and root microbial communities in our system are dominated by variable selection (βNTI index >2). Observing a βNTI index lower than 2 would indicate that plants will assemble similar microbial communities starting from different inocula (homogenous selection). This observation, although interesting, might simply be generated by the use of different inocula, which generate more dissimilar plant-associated microbial communities. This is not a surprising result, as it is widely acknowledged that plants growing in soils hosting different microbial communities are associated with dissimilar microbial communities. On the other hand, plants are continuously challenged by growing on soils hosting very dissimilar microbial communities, including spatial variation across small (seed dispersal) and large scales (seed commercialization), temporal variation at small (within the same year) or large scales (across multiple years, e.g., soil seed banks), plant-soil feedback, plant range expansion, and several other events. Although plants might be associated with different microbial taxa in different contexts, they might still exert selective pressure on function rather than identity, as microbial communities with different structures might code for similar functions (Doolittle and Booth, 2017). Several studies have suggested that plants can modulate their microbiome through changes in root exudates and VOCs (Badri et al., 2013; Chaparro et al., 2013; Tiziani et al., 2022), including the selection of specific microbial functions that might differ throughout plant development (Chaparro et al., 2013). This mechanism might explain why the same plant genotype grown in association with different microbial communities might have little phenotypic variation. This is an interesting perspective that needs to be addressed by multi-omics studies that go beyond the taxonomic composition of the microbial community and focus on its functional role in the host plant. In addition to the idea that the selection of dissimilar microbiomes might be entirely driven by the host plant, we also need to consider that different soil microbial communities might be characterized by different interactions between the members of the microbiome, which in turn might influence the final outcome of plant-microbiome interactions (Hassani et al., 2018).

Interestingly, we observed only marginal differences in the structure of the microbial communities between the shoots and roots. This contrasts with the general idea that plant-associated microbial communities are mainly assembled by organ or compartment (Trivedi et al., 2020), and there is evidence that this differentiation can also be detected in gnotobiotic conditions (Abdelfattah et al., 2021; Wang et al., 2020). However, our study focused on seedlings at the early developmental stage, whereas previous studies mainly focused on plants at a later developmental stage. This might explain the inconsistency of the results, suggesting that the shoot microbiome is first assembled from the soil/-root microbiome, but then differentiated through microbial recruitment or transient association with microorganisms from the environment. Future studies may provide more detailed evidence for this idea. In addition, when focusing on gnotobiotic plants not exposed to inocula, we observed a community composed of 172 bacterial and 9 fungal ASVs that were highly variable at the level of individual seedlings. This is consistent with results from previous studies on the microbiome of single seeds, which showed a very high variability in the composition of the microbial community between individual seeds (Chesneau et al., 2022; Kim et al., 2023).

Our study contributes to expanding our understanding of the mechanisms that guide the assembly of the plant microbiome, suggesting that plants can drive selection processes at early developmental stages. Although this idea needs to be tested with a wider set of hosts and under different conditions, it provides further evidence that will help clarify patterns in the assembly of microbial communities across plant species. This information is key to understanding the functioning of the plant microbiome and how we can direct its assembly to influence the host or guide us in the assembly of synthetic microbial communities that can help achieve more sustainable agriculture and ecosystem restoration.

CRediT authorship contribution statement

Nesma Zakaria Mohamed: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Leonardo Schena:** Writing – review & editing, Funding acquisition, Conceptualization. **Antonino Malacrinò:** Writing – review & editing, Visualization, Methodology, Investigation, Conceptualization.

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.

Data availability

Raw sequencing data are available on the NCBI SRA under Bioprojects PRJNA1029922 (16S) and PRJNA1029924 (ITS). Code is available at: https://github.com/amalacrino/gnotobiotic_lettuce.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2024.100896.

References

Abdelfattah, A., Sanzani, S.M., Wisniewski, M., Berg, G., Cacciola, S.O., Schena, L., 2019. Revealing cues for fungal interplay in the plant-air interface in vineyards. Front. Plant Sci. 10, 922.

Abdelfattah, A., Wisniewski, M., Schena, L., Tack, A.J.M., 2021. Experimental evidence of microbial inheritance in plants and transmission routes from seed to phyllosphere and root. Environ. Microbiol. 23, 2199–2214.

Arnault, G., Mony, C., Vandenkoornhuyse, P., 2022. Plant microbiota dysbiosis and the Anna Karenina principle. Trends Plant Sci. https://doi.org/10.1016/j.tplants.2022.08.012.

Badri, D.V., Chaparro, J.M., Zhang, R., Shen, Q., Vivanco, J.M., 2013. Application of natural blends of phytochemicals derived from the root exudates of *Arabidopsis* to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. J. Biol. Chem. 288, 4502–4512.

Badri, D.V., Vivanco, J.M., 2009. Regulation and function of root exudates. Plant Cell Environ. 32, 666–681.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Software 67, 1–48.

Bell, J.K., Mamet, S.D., Helgason, B., Siciliano, S.D., 2022. *Brassica napus* bacterial assembly processes vary with plant compartment and growth stage but not between lines. Appl. Environ. Microbiol. 88, e0027322.

Berg, G., Kusstatscher, P., Abdelfattah, A., Cernava, T., Smalla, K., 2021. Microbiome modulation-toward a better understanding of plant microbiome response to microbial inoculants. Front. Microbiol. 12, 650610.

Cai, Z., Wang, X., Bhadra, S., Gao, Q., 2020. Distinct factors drive the assembly of quinoa-associated microbiomes along elevation. Plant Soil 448, 55–69.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 6, 1621–1624.

Chaparro, J.M., Badri, D.V., Bakker, M.G., Sugiyama, A., Manter, D.K., Vivanco, J.M., 2013. Root exudation of phytochemicals in *Arabidopsis* follows specific patterns that are developmentally programmed and correlate with soil microbial functions. PLoS One 8, e55731.

Chen, S., Wang, L., Gao, J., Zhao, Y., Wang, Y., Qi, J., Peng, Z., Chen, B., Pan, H., Wang, Z., Gao, H., Jiao, S., Wei, G., 2022. Agricultural management drive bacterial community assembly in different compartments of soybean soil-plant continuum. Front. Microbiol. 13, 868307.

Chesneau, G., Laroche, B., Préveaux, A., Marais, C., Briand, M., Marolleau, B., Simonin, M., Barret, M., 2022. Single seed microbiota: assembly and transmission from parent plant to seedling. mBio 13, e0164822.

Davis, N.M., Proctor, D.M., Holmes, S.P., Relman, D.A., Callahan, B.J., 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6, 226.

Davoudpour, Y., Schmidt, M., Calabrese, F., Richnow, H.H., Musat, N., 2020. High resolution microscopy to evaluate the efficiency of surface sterilization of *Zea mays* seeds. PLoS One 15, e0242247.

Dini-Andreote, F., Raaijmakers, J.M., 2018. Embracing community ecology in plant microbiome research. Trends Plant Sci. 23, 467–469.

Dini-Andreote, F., Stegen, J.C., van Elsas, J.D., Salles, J.F., 2015. Disentangling mechanisms that mediate the balance between stochastic and deterministic processes in microbial succession. Proc. Natl. Acad. Sci. U.S.A. 112, E1326–E1332.

Dixon, P., 2003. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14, 927–930.

Doolittle, W.F., Booth, A., 2017. It's the song, not the singer: an exploration of holobiosis and evolutionary theory. Biol. Philos. 32, 5–24.

Dove, N.C., Veach, A.M., Muchero, W., Wahl, T., Stegen, J.C., Schadt, C.W., Cregger, M. A., 2021. Assembly of the *Populus* microbiome is temporally dynamic and determined by selective and stochastic factors. mSphere 6, e0131620.

Fox, J., Weisberg, S., 2018. An R Companion to Applied Regression. SAGE Publications.

Francis, J.S., Mueller, T.G., Vannette, R.L., 2023. Intraspecific variation in realized dispersal probability and host quality shape nectar microbiomes. New Phytol. 240, 1233–1245.

Fu, S., Deng, Y., Zou, K., Zhang, S., Duan, Z., Wu, X., Zhou, J., Li, S., Liu, X., Liang, Y., 2023. Dynamic variation of *Paris polyphylla* root-associated microbiome assembly with planting years. Planta 257, 61.

Gao, C., Montoya, L., Xu, L., Madera, M., Hollingsworth, J., Purdom, E., Singan, V., Vogel, J., Hutmacher, R.B., Dahlberg, J.A., Coleman-Derr, D., Lemaux, P.G., Taylor, J.W., 2020. Fungal community assembly in drought-stressed sorghum shows stochasticity, selection, and universal ecological dynamics. Nat. Commun. 11, 34.

Guo, J., Ling, N., Li, Y., Li, K., Ning, H., Shen, Q., Guo, S., Vandenkoornhuyse, P., 2021. Seed-borne, endospheric and rhizospheric core microbiota as predictors of plant functional traits across rice cultivars are dominated by deterministic processes. New Phytol. 230, 2047–2060.

Hassani, M.A., Durán, P., Hacquard, S., 2018. Microbial interactions within the plant holobiont. Microbiome 6, 58.

Hervé, M., 2022. RVAideMemoire: Testing and Plotting Procedures for Biostatistics.

Kembel, S.W., Cowan, P.D., Helmus, M.R., Cornwell, W.K., Morlon, H., Ackerly, D.D., Blomberg, S.P., Webb, C.O., 2010. Picante: R tools for integrating phylogenies and ecology. Bioinformatics 26, 1463–1464.

Kim, H., Kim, C., Lee, Y.-H., 2023. The single-seed microbiota reveals rare taxa-associated community robustness. Phytobiomes J. https://doi.org/10.1094/pbiomes-10-22-0068-r.

Kuang, L., Li, T., Wang, B., Peng, J., Li, J., Li, P., Jiang, J., 2023. Diseased-induced multifaceted variations in community assembly and functions of plant-associated microbiomes. Front. Microbiol. 14, 1141585.

Kumar, M.S., Slud, E.V., Okrah, K., Hicks, S.C., Hannenhalli, S., Corrada Bravo, H., 2018. Analysis and correction of compositional bias in sparse sequencing count data. BMC Genom. 19, 799.

Larsen, S., Albanese, D., Stegen, J., Franceschi, P., Coller, E., Zanzotti, R., Ioriatti, C., Stefani, E., Pindo, M., Cestaro, A., Donati, C., 2023. Distinct and temporally stable assembly mechanisms shape bacterial and fungal communities in vineyard soils. Microb. Ecol. 86, 337–349.

Lenth, R.V., 2022. emmeans: Estimated Marginal Means, Aka Least-Squares Means.

Louisson, Z., Ranjard, L., Buckley, H.L., Case, B.S., Lear, G., 2023. Soil bacterial community composition is more stable in kiwifruit orchards relative to phyllosphere communities over time. Environ. Microbiome 18, 71.

Maignien, L., DeForce, E.A., Chafee, M.E., Eren, A.M., Simmons, S.L., 2014. Ecological succession and stochastic variation in the assembly of *Arabidopsis thaliana* phyllosphere communities. mBio 5, e00682, 13.

Malacrinò, A., Abdelfattah, A., Berg, G., Benitez, M.-S., Bennett, A.E., Böttner, L., Xu, S., Schena, L., 2022. Exploring microbiomes for plant disease management. Biol. Control 169, 104890.

Malacrinò, A., Karley, A.J., Schena, L., Bennett, A.E., 2021. Soil microbial diversity impacts plant Microbiota more than herbivory. Phytobiomes J. 5, 408–417.

Mallick, H., Rahnavard, A., McIver, L.J., Ma, S., Zhang, Y., Nguyen, L.H., Tickle, T.L., Weingart, G., Ren, B., Schwager, E.H., Chatterjee, S., Thompson, K.N., Wilkinson, J. E., Subramanian, A., Lu, Y., Waldron, L., Paulson, J.N., Franzosa, E.A., Bravo, H.C., Huttenhower, C., 2021. Multivariable association discovery in population-scale meta-omics studies. PLoS Comput. Biol. 17, e1009442.

McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217.

Mittelstrass, J., Sperone, F.G., Horton, M.W., 2021. Using transects to disentangle the environmental drivers of plant-microbiome assembly. Plant Cell Environ. 44, 3515–3525.

Moroenyane, I., Mendes, L., Tremblay, J., Tripathi, B., Yergeau, É., 2021. Plant compartments and developmental stages modulate the balance between niche-based and neutral processes in soybean microbiome. Microb. Ecol. 82, 416–428.

Nilsson, R.H., Larsson, K.-H., Taylor, A.F.S., Bengtsson-Palme, J., Jeppesen, T.S., Schigel, D., Kennedy, P., Picard, K., Glöckner, F.O., Tedersoo, L., Saar, I., Kõljalg, U., Abarenkov, K., 2019. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. Nucleic Acids Res. 47, D259–D264.

Ning, D., Yuan, M., Wu, L., Zhang, Y., Guo, X., Zhou, X., Yang, Y., Arkin, A.P., Firestone, M.K., Zhou, J., 2020. A quantitative framework reveals ecological drivers of grassland microbial community assembly in response to warming. Nat. Commun. 11, 4717.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596.

R Core Team, 2020. R: A Language and Environment for Statistical Computing.

Rochefort, A., Simonin, M., Marais, C., Guillerm-Erckelboudt, A.-Y., Barret, M., Sarniguet, A., 2021. Transmission of seed and soil microbiota to seedling. mSystems 6, e0044621.

Schliep, K.P., 2011. phangorn: phylogenetic analysis in R. Bioinformatics 27, 592–593.

Shade, A., Jacques, M.-A., Barret, M., 2017. Ecological patterns of seed microbiome diversity, transmission, and assembly. Curr. Opin. Microbiol. 37, 15–22.

Song, C., Zhu, F., Carrión, V.J., Cordovez, V., 2020. Beyond plant microbiome composition: exploiting microbial functions and plant traits via integrated approaches. Front. Bioeng. Biotechnol. 8, 896.

Sudarshan, L., Shetty, L., 2017. Tools for Microbiome Analysis in R.

Tiziani, R., Miras-Moreno, B., Malacrinò, A., Vescio, R., Lucini, L., Mimmo, T., Cesco, S., Sorgonà, A., 2022. Drought, heat, and their combination impact the root exudation patterns and rhizosphere microbiome in maize roots. Environ. Exp. Bot. 203, 105071.

Toju, H., Tanabe, A.S., Yamamoto, S., Sato, H., 2012. High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. PLoS One 7, e40863.

Trivedi, P., Leach, J.E., Tringe, S.G., Sa, T., Singh, B.K., 2020. Plant–microbiome interactions: from community assembly to plant health. Nat. Rev. Microbiol. 18, 607–621.

Walsh, C.M., Becker-Uncapher, I., Carlson, M., Fierer, N., 2021. Variable influences of soil and seed-associated bacterial communities on the assembly of seedling microbiomes. ISME J. 15, 2748–2762.

Wang, L., Liu, J., Zhang, M., Wu, T., Chai, B., 2023. Ecological processes of bacterial and fungal communities associated with *Typha orientalis* roots in wetlands were distinct during plant development. Microbiol. Spectr. 11, e0505122.

Wang, M., Eyre, A.W., Thon, M.R., Oh, Y., Dean, R.A., 2020. Dynamic changes in the microbiome of rice during shoot and root growth derived from seeds. Front. Microbiol. 11, 559728.

Wang, Z., Wang, H., Chen, Z., Wu, Q., Huang, K., Ke, Q., Zhu, L., Lu, S., Tang, Y., Li, H., Chen, L., Wu, L., 2022. Ecological niche differences regulate the assembly of bacterial community in endophytic and rhizosphere of *Eucalyptus*. For. Ecol. Manage. 524, 120521.

Wickham, H., 2009. ggplot2: Elegant Graphics for Data Analysis. Springer, New York, NY.

Wright, E.S., 2015. DECIPHER: harnessing local sequence context to improve protein multiple sequence alignment. BMC Bioinf. 16, 322.

Xiong, C., Singh, B.K., He, J.-Z., Han, Y.-L., Li, P.-P., Wan, L.-H., Meng, G.-Z., Liu, S.-Y., Wang, J.-T., Wu, C.-F., Ge, A.-H., Zhang, L.-M., 2021. Plant developmental stage drives the differentiation in ecological role of the maize microbiome. Microbiome 9, 171.

Yan, Z.-Z., Chen, Q.-L., Li, C.-Y., Thi Nguyen, B.-A., He, J.-Z., Hu, H.-W., 2022. Contrasting ecological processes shape the *Eucalyptus* phyllosphere bacterial and fungal community assemblies. J of Sust Agri & Env 1, 73–83.

Yin, Y., Wang, Y.-F., Cui, H.-L., Zhou, R., Li, L., Duan, G.-L., Zhu, Y.-G., 2023. Distinctive structure and assembly of phyllosphere microbial communities between wild and cultivated rice. Microbiol. Spectr. 11, e0437122.