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A gnotobiotic-based approach to study the plant microbiome assembly

PH.D. THESIS

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Abstract

The current challenges in agricultural production, such as a growing global population, environmental impacts of climate change, and constraints on land and water resources, need the urgent exploration and implementation of innovative sustainable solutions. Ensuring food security requires addressing these issues comprehensively. Consequently, the investigation of the plant microbiome has gained importance due to its potential to enhance plant growth in adverse conditions. Even though plant microbiomes play an essential role in agricultural sustainability, numerous questions regarding their assembly and impact on plant fitness, ecology, and evolution remain unanswered. The goal of this project is to use gnotobiotic plants (plants with low number of associated microorganisms) and metagenomics techniques to decipher the complicated network of relationships between hosts and their microbiomes. Our results contribute to a better understanding of plant-microbiome dynamics and their effect on plant ecology and evolution, with important potential application in sustainable agriculture and resilience to climate changes.

We first conducted a systematic review of existing literature on the plant microbiome, aiming to highlight knowledge gaps and identify technical issues. Our analysis involved collecting data from 610 studies, focusing on DNA extraction methods, primer pairs, sequencing technologies, and data availability. The findings revealed significant variability in approaches across articles, posing challenges for meaningful comparisons between studies and emphasizing the need for standardized methodologies in plant microbiome research.

Second, using gnotobiotic lettuce plants inoculated with diverse soil samples, we aimed at understanding how plant microbiomes are assembled during early developmental stages. Results from our study show that host plants do not initially exert a selective influence on the microbiome of gnotobiotic plants (those inherited from seeds). However, this dynamic shifts rapidly when plants come into contact with soil microbiomes. In just one week, plants demonstrated the ability to selectively choose microorganisms from the inocula, assemble the root microbiome and from this, assemble the shoot microbiome through deterministic processes.

Third, we aimed to select a microbial community that can effectively contrast the development of postharvest diseases. This was achieved by subjecting microbial communities obtained from five different sources to selective pressure over ten cycles on apple fruits. We then used the microbial community from the first and tenth generation to inoculate apple fruits together with two pathogenic fungi: *Botrytis cinerea* and *Penicillium expansum*. Our findings indicated a significant change in the structure of microbial communities over successive cycles, and resulting communities were able to reduce disease by 90% (*B. cinerea*) and 70% (*P. expansum*).

Fourth, we investigated how increases in environmental temperature affect plant-microbiome interactions by simulating global warming scenarios and the subsequent effect on plant phenotypic plasticity. We used gnotobiotic *Spirodela polyrhiza* as a model species, inoculated 99 genotypes with a synthetic bacterial community, and cultivated plants at two different temperatures. Our results showed that temperature, population, and time point significantly impacted changes in plant phenotypic traits such as surface area, dry biomass, and fronds reproduction rates. This effect can potentially influence the evolution of plant populations under higher temperatures.

Keywords: plant microbiome, systematic review, amplicon metagenomics, postharvest disease management, global warming, deterministic and stochastic mechanisms

Riassunto

Le attuali sfide nella produzione agricola, come la crescita della popolazione globale, gli impatti dei cambiamenti climatici e i vincoli sulle risorse terrestri e idriche, richiedono l'urgente esplorazione e implementazione di soluzioni sostenibili innovative. Lo studio del microbioma vegetale ha acquisito importanza grazie al suo potenziale di migliorare la crescita delle piante in condizioni avverse. Anche se i microbiomi vegetali svolgono un ruolo essenziale nella sostenibilità agricola, numerose domande riguardanti il loro assemblaggio e il loro impatto sulla salute, sull'ecologia e sull'evoluzione delle piante rimangono senza risposta. L'obiettivo di questa tesi è stato quello di utilizzare piante gnotobiotiche (piante con un basso numero di microrganismi associati) per decifrare la complicata rete di relazioni tra ospiti e i loro microbiomi. I nostri risultati contribuiscono a una migliore comprensione delle dinamiche pianta-microbioma e del loro effetto sull'ecologia e sull'evoluzione delle piante, con importanti potenzialità di applicazione nell'agricoltura sostenibile e resilienza ai cambiamenti climatici.

Per prima cosa abbiamo condotto una revisione sistematica della letteratura esistente sul microbioma vegetale, con l'obiettivo di evidenziare le lacune nelle conoscenze e identificare le questioni tecniche. La nostra analisi condotta raccogliendo dati da 610 studi ha rivelato una variabilità significativa negli approcci per lo studio del microbioma delle piante, ponendo sfide per confronti significativi tra gli studi e sottolineando la necessità di metodologie standardizzate nella ricerca sul microbioma vegetale.

In secondo luogo, utilizzando piante gnotobiotiche abbiamo mirato a comprendere come vengono assemblati i microbiomi vegetali durante le prime fasi di sviluppo. I risultati del nostro studio mostrano che le piante ospiti inizialmente non esercitano un'influenza selettiva sul microbioma delle piante gnotobiotiche (quello ereditato dai semi). Tuttavia, questa dinamica cambia rapidamente quando le piante entrano in contatto con il microbioma del suolo. In una sola settimana, le piante hanno dimostrato la capacità di scegliere selettivamente i microrganismi dagli inoculi, assemblare il microbioma della radice e da questo assemblare il microbioma del germoglio attraverso processi deterministici.

In terzo luogo, abbiamo mirato a selezionare una comunità microbica in grado di contrastare efficacemente lo sviluppo di malattie post-raccolta. Ciò è stato ottenuto sottoponendo le comunità microbiche ottenute da cinque diverse fonti a pressione selettiva. Abbiamo poi utilizzato la comunità microbica della prima e della decima generazione per inoculare mele insieme a due funghi patogeni: *Botrytis cinerea* e *Penicillium expansum*. I nostri risultati hanno indicato un cambiamento significativo nella struttura delle comunità microbiche nel corso dei cicli successivi e le comunità risultanti sono state in grado di ridurre la malattia del 90% (*B. cinerea*) e del 70% (*P. expansum*).

In quarto luogo, abbiamo studiato come gli aumenti della temperatura ambientale influenzano le interazioni pianta-microbioma simulando scenari di riscaldamento globale e il conseguente effetto sulla plasticità fenotipica delle piante. Abbiamo utilizzato *Spirodela polyrhiza* come specie modello, inoculato 99 genotipi gnotobiotici con una comunità batterica sintetica e coltivato piante a due diverse temperature. I nostri risultati hanno mostrato che la temperatura, la popolazione e il punto temporale hanno avuto un impatto significativo sui cambiamenti nei tratti fenotipici delle piante come la superficie, la biomassa secca e i tassi di riproduzione delle fronde. Questo effetto può potenzialmente influenzare l'evoluzione di comunità di piante a temperature più elevate.

Parole chiave: microbioma vegetale, revisione sistematica, metagenomica degli ampliconi, gestione delle malattie post-raccolta, riscaldamento globale, meccanismi deterministici e stocastici

Chapter 1. General Introduction

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1.1. Problem and challenges facing agriculture production

In recent years, agriculture has faced numerous challenges. These issues can differ based on geographical location and may change over time because of the unique climate and soil characteristics in each region. The main obstacle that has had a significant and ongoing effect on agricultural productivity is the worldwide increase in population. Estimates suggest that the global population will increase to 9.2 billion in 2050 (Pawlak and Kołodziejczak, 2020). Most of this growth will focus on developing nations, specifically in the world's least developed economies, where a relative increase of 120% is expected (Hossain *et al*., 2020). The demand for food is expected to rise by 59–102% because of two factors: the growing global population (Pawlak and Kołodziejczak, 2020) and the trend towards a diet higher in protein, which includes meat and dairy products, rather than starchy foods (Baldos and Hertel, 2014). Consequently, in the coming years, there will be a significant increase in the consumption of crop output, which includes both direct consumption and feed for the livestock (Baldos and Hertel, 2014).

Greenhouse gas emissions, which in turn cause higher levels of climate change, are strongly correlated with increased livestock production (Cheng *et al*., 2022). The devastating effects of climate change on the Earth's ecosystems make it one of the most pressing problems in today's world. The average global temperature has increased by 0.9 °C in the last century, largely as a result of human activities, which have also significantly accelerated the rate of climate change. Due to severe soil, water, and air pollution, as well as increasing greenhouse gas emissions, experts predict that global temperatures will continue to rise, reaching a global average increase of 1.5 °C or more by the year 2050 (Arora, 2019). Worldwide, heatwaves, droughts, floods, and irregular precipitation patterns have increased in frequency and severity due to the extraordinary rise in global temperatures (Abbass *et al*., 2022). Global heat stress has resulted in significant crop losses worldwide. China has experienced a loss of approximately 5.18 million tons of rice over the past decade. In Southeast Asia, this percentage has reached as high as 14%. In 2001, heat stress affected approximately 7 million hectares of wheat in developing countries and 36 million hectares in temperate regions, resulting in a reduction of 19 million tons in yield (Ahmad *et al*., 2022).

An additional crucial aspect of climate change and global warming is the wider spread of plant pathogens and the incidence of plant diseases in the field. Elevated temperatures can influence the population dynamics of pathogens, influencing their ability to survive winter, persist, and increase in numbers (Singh *et al*., 2023; Hulme, 2017). This can lead to an increase in the number of generations of polycyclic species, thereby enhancing the transmission and effects of plant pathogens. Moreover, elevated temperatures can reduce the duration of pathogen incubation, leading to an increased incidence of diseases throughout the growing season (Singh *et al*., 2023). Climate change can have indirect effects on plant-pathogen interactions by modifying plant architecture and subsequently affecting the microenvironment, in addition to its direct effects (Elad and Pertot, 2014). Modifications in the biochemical, physiological, ecological, and evolutionary

processes of the host plant, environmental conditions, and pathogen can alter the dynamics of their interactions. For example, more frequent and intense extreme weather events caused by climate change can help pathogens to spread to new areas. This was apparent in the case of soybean rust, which was introduced into the United States from Brazil by means of a hurricane (Singh *et al*., 2023). These changes may result in more severe infections and alter both pathogen diversity and host resistance to diseases. To address this problem, farmers depend only on synthetic chemicals, such as pesticides, to reduce or prevent pathogen attacks and increase plant production. Although pesticides have a positive impact on plant production, they have been shown to have multiple drawbacks in terms of both environmental and human health (Wilson and Tisdell, 2001; Tudi *et al*., 2021). To address these challenges, it is crucial to investigate alternative strategies that can reduce the negative effects of climate change on the interactions between plants and pathogens, while also promoting sustainable agricultural practices.

Exploration of plant microbiomes has emerged as a promising avenue for addressing these challenges. Harnessing the natural interactions between plants and beneficial microbes has the potential to enhance plant resilience, mitigate the effects of climate change, and reduce the dependence on synthetic chemicals. By leveraging the complex relationships within the plant microbiome, resilient agricultural ecosystems that are adaptable to changing environmental conditions can be developed. This shift towards holistic and sustainable practices not only aligns with the principles of ecological balance but also fosters long-term agricultural sustainability in the face of evolving global challenges. As we progress into the future, the investigation of plant microbiomes offers a revolutionary method that shows the potential for promoting sustainable and resilient agricultural systems.

1.2. Importance of the plant microbiome and factors behind its assembly

The "plant microbiome" is a term commonly used when referring to communities of microorganisms tightly associated with plants, including bacteria, fungi, protozoa, archaea, and others (Tosi *et al*., 2020; Trivedi *et al*., 2022). These communities can be classified according to the specific plant compartments they inhabit, for example into phyllosphere (the above-ground part of the plant), endosphere (the interior of plant tissue), epiphytes (the plant's surface), and rhizosphere (the soil zone directly surrounding the roots, influenced by root exudates) (Gupta *et al*., 2021; Vishwakarma *et al*., 2020). The plant microbiome has a wide range of abilities, from pathogenicity to neutrality to beneficial effects on plant development and growth. These beneficial effects can be achieved for example directly by regulating the production of growth hormones and enhancing nutrient uptake and growth, and/or indirectly by suppressing plant pathogens and inducing plant systemic resistance (Olanrewaju *et al*., 2017; Trivedi *et al*., 2020). Microbial communities can be transmitted to the host plants by two mechanisms: either horizontally from the surrounding environment or vertically from the parental plants to the offspring (fruits and seeds) (Shade *et al*., 2017; Berg *et al*., 2021). The endophytes present in seeds contribute to the process of germination and the first development of plants (Shahzad *et al*., 2018). Moreover, seeds initiate the formation of a spermosphere area by releasing several metabolites that initiate horizontal transmission by attracting microbial communities from nearby soil to colonize the seed tissue. Once established, these microorganisms can migrate to other parts of the plant, such as roots, leaves, and flowers.

Eventually, they return to the seeds to complete their life cycle, forming a continuous and interwoven symbiotic interaction between the seeds and their associated microbial populations (Santos and Olivares, 2021).

Research has shown that plant microbiomes are not random collections of organisms but are driven by broad principles directed by complex interactions between microorganisms, their host plant, and the environment (Trivedi *et al*., 2020). Their assembly and functional structure can be shaped and altered by a variety of biotic and abiotic factors. These factors are linked to pathogen attacks, implementation of specific agricultural practices, various environmental conditions, and the plant itself (Santos and Olivares, 2021). Numerous studies have revealed that in response to biotic or abiotic stressors, plants have evolved an exudation-mediated response to help, which results in the release of specific metabolites and the recruitment of a stress-relieving microbiome (Gao *et al*., 2021; Trivedi *et al*., 2022). Furthermore, environmental factors such as soil (e.g., structure, pH, C/N ratio) and climatic conditions (e.g., temperature, humidity, UV radiation) can alter the composition of plant-associated microbial communities (Dastogeer *et al*., 2020). According to several studies, the rhizospheric microbiome of plants varies according to their developmental stages (seedling, vegetative, and flowering), as well as the type of tissue that they colonize (Santoyo, 2022; Schreiter *et al*., 2014). Understanding the factors that contribute to plant microbiome assembly and the discovery of beneficial microbes within these communities could be crucial to the development of sustainable agriculture and increase in plant productivity (Mittelstrass *et al*., 2021).

1.3. Systematic review study about plant microbial communities and metagenomic approaches

A large body of literature on plant microbiomes emphasizes the significance of this topic. These studies investigated various plant compartments across the soil-plant continuum, demonstrating how environmental factors modulate microbiome changes, and proposed promising agricultural applications (Santos and Olivares, 2021). Considering the high number of publications generated every year on plant-microbiome interactions, we performed a systematic review of the literature to highlight knowledge gaps and technical issues that are preventing the field from growing further.

A search for the term "plant AND (microbiota OR microbiome)" in the SCOPUS database covering the years 2011-2021 yielded 117,579 papers (November 23, 2021). For this first analysis, we restricted our search to the year 2021, yielding a total of 1,928 articles (November 23, 2021). Two filtration steps were conducted, the first of which involved reading the title of each article, eliminating any papers that were not relevant to the study, along with the review articles on plant microbiomes. A second filtration process was conducted to exclude papers that did not utilize amplicon or shotgun metagenomics to infer the plant microbiome, yielding 610 studies. The following metadata were collected for each study: DNA extraction method, metagenomic approach (shotgun or amplicon, DNA or RNA), primer pairs used, sequencing technology, data public availability, plant species, and compartment.

We identified 109 distinct methods for DNA extraction. Additionally, there were 25 primer pairs targeting 16S rRNA and 12 primer pairs targeting ITS rRNA. These were used in conjunction

with seven different sequencing technologies. The MoBio PowerSoil DNA Isolation kit was used in the majority of studies (*n* = 82), followed by the QIAGEN DNeasy PowerSoil kit (*n* = 77), and the MOBIO PowerSoil Kit (*n* = 61). The primer pair 515F-806R was the most commonly used for 16S rRNA amplification (*n* = 146), whereas for ITS, the primer pair ITS1-ITS2 was extensively employed (*n* = 124). The resulting amplicons were sequenced using Illumina technology with a total of 567 studies. Among these, 429 studies sequenced samples on the MiSeq platform, whereas 86 studies used the HiSeq platform. Seventeen studies have reported the use of PNA primers to stop plant DNA amplification. Most data (*n* = 400) were submitted to the SRA database. A total of 169 distinct plant species were examined in the studies, most of which focused on microbial communities collected from bulk soil (*n* = 201), multiple compartments (*n* = 176), and the rhizosphere (*n* = 135). In addition, the majority of studies examined a single time point (*n* = 479) rather than multiple time points (*n* = 126). On the other hand, multiple studies neglected to include crucial details necessary for replication, such as the method used for extracting DNA, the specific primer pairs employed, and the sequencing platform utilized (*n* = 15, 32, and 171, respectively).

Because of the diversity of methods used to investigate plant microbiomes in various settings, it can be challenging to draw meaningful comparisons between studies. Notably, DNA extraction methods play a pivotal role in influencing the outcomes of metabarcoding analyses, as highlighted in various studies (McOrist *et al*., 2002; Walker *et al*., 2015; Vasselon *et al*., 2017; Hallmaier-Wacker *et al*., 2018). The final reconstruction of the microbial community can be influenced by diverse methods owing to variations in the efficiency of DNA recovery or the ability of the protocols to isolate DNA from specific cell groups, such as gram-positive bacteria. Furthermore, various DNA extraction methods may have different levels of effectiveness in removing PCR inhibitors, which are critical factors affecting the quality of amplicon libraries. Nevertheless, no single study has established a direct relationship between specific DNA extraction techniques and the presence or absence of distinct bacterial groups. As a result, it is not possible to accurately predict which taxa may be over- or under-represented using different protocols (Malacrinò, 2022).

Although it is a significant challenge to standardize DNA extraction for the entire field, this should be suggested for PCR primer pairs. Previous studies by Bahram *et al*., (2019), Parada *et al*., (2016), and Tremblay *et al*., (2015) established that primer pairs can impact the results of metabarcoding analyses. Another study focused on the ability of primer pairs to reconstruct mock communities and discovered that some primers performed better than others, and that some failed to amplify entire microbial groups (Abellan-Schneyder *et al*., 2021). Therefore, when dealing with new systems, it is becoming increasingly necessary to conduct preliminary tests using various primer combinations. However, this limitation could be overcome by sequencing the entire 16S rRNA, for example, by using PacBio or Oxford Nanopore sequencing technologies. Our findings indicate that 171 studies failed to provide raw data, thereby reducing their reproducibility, and limiting comparisons with other studies during statistical approaches such as meta-analysis.

To summarize, this study has provided insights into several factors that can have a substantial impact on the resulting data when studying plant microbiomes, including the techniques used for DNA extraction and the specific primers employed. Comprehensive planning is essential for choosing a suitable approach to ensure that the ultimate data remains comparable and replicable

in the future. To obtain a more comprehensive understanding of plant microbiome studies, a larger systematic review and meta-analysis conducted over multiple years is required.

1.4. Gnotobiotic plants and their importance in studying plant microbiome

As we explore the complexities of plant microbiome field further, more questions frequently emerge. For instance, how do these microbiomes interact with their plant hosts, and what role do these interactions play in the overall health and productivity of the plant? Can we use all this information to manipulate the plant microbiome to enhance plant growth and health? It is essential to tackle these questions as we endeavor to exploit the potential of plant microbiomes for sustainable agriculture. The utilization of plant microbiomes could offer significant benefits in enhancing crop productivity and resilience. However, before we can fully exploit these benefits, we must first understand the complex interplay between plant microbiomes and their hosts. We need to unravel the intricate mechanisms through which these microbiomes function, and how they respond to the challenges posed by climate change. These questions represent just a small fraction of the many unanswered questions in the field of plant microbiomes.

Gnotobiotic organisms represent an excellent model to answer these questions. The phrase "gnotobiotic" comes from the Greek terms "gnotos" and "biota," translating to "known life" or "known organisms" (Basic and Bleich, 2019). A gnotobiotic organism is a model organism that is colonized by a specific community of known microorganisms or includes no microorganisms (germ-free) and is frequently used for experimental purposes (Gordon and Pesti, 1971). This method is critical for investigating plant-microbiome interactions because it reduces complexity, increases reproducibility, and allows for precise manipulation of the plant's microbial community (Ma *et al*., 2022).

Plant gnotobiotic systems have been extensively employed in previous studies to gain insights into plant-microbiome relationships. For example, plant gnotobiotic systems have been utilized to investigate the role of root microbiota in plant health and growth under adverse environmental conditions by inoculating plants with growth-promoting endophytic bacteria (Molina *et al*., 2021). Another study employed gnotobiotic plants of *Arabidopsis thaliana* to investigate the effectiveness of synthetic bacterial communities composed of five strains in protecting the plant against *Pseudomonas syringae* (Emmenegger *et al*., 2023). Essentially, gnotobiotic plants play a crucial role in microbiome studies by allowing researchers to prove causal relationships between the genotypes and phenotypes of plants and bacteria, thus enhancing our understanding of plant-microbiome interactions.

The objective of this study was to use a gnotobiotic approach to construct three models with different host plants to tackle agricultural production challenges and address unanswered questions regarding the plant microbiome.

1- First, we aimed at understanding the assembly process of plant microbiomes in the early developmental stages by using gnotobiotic lettuce plants inoculated with different soil inocula under controlled conditions. We hypothesize that deterministic processes will guide the assembly of seedling roots and shoots after soil inoculation.

2- Second, using apple fruits as a model system, we investigate the possibility of establishing a stable microbial community with antagonistic effects against postharvest disease. This study is conducted by using epiphytic microbial communities from five plant sources and multigenerational techniques. We hypothesize that by subjecting microbiomes to selection pressure over ten cycles of rapid reinoculation, we will select a microbial community capable of rapidly colonizing wounds and protect fruits from the development of rots.
3- Third, we examine the impact of global warming and temperature changes on plant microbiome interactions. This was accomplished by growing 99 different genotypes of *Spirodela polyrhiza* that were inoculated with a bacterial synthetic community, all under the influence of two temperatures. Our hypothesis is that the host genotype-microbiome interactions will alter the plant phenotype with an effect dependent on environmental temperatures.

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Chapter 2. Lettuce seedlings rapidly assemble their microbiome from the environment through deterministic processes

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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.

Keywords: gnotobiotic plants; amplicon metagenomics; 16S; ITS; ecological processes

1. 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 (Song *et al*., 2020; Trivedi *et al*., 2020; Malacrinò *et al*., 2022). 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 (Shade *et al*., 2017; Berg *et al*., 2021; Abdelfattah *et al*., 2019). 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 (Xiong *et al*., 2021; Maignien *et al*., 2014) or as an effect of stress (Kuang *et al*., 2023; Gao *et al*., 2020). 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 (Xiong *et al*., 2021; Maignien *et al*., 2014; Dini-Andreote and Raaijmakers, 2018) 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 (Xiong *et al*., 2021; Wang *et al*., 2023), 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 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 whether seedlings create a selective environment belowground and, through selection, drive the assembly of root and leaf microbiomes through deterministic processes. We hypothesized that seedlings create a selective environment belowground, and that through deterministic processes, they assemble both root and shoot microbiomes. 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.

2. Materials and methods

2.1. 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 autoclaved soil was prepared by sieving the soil to 1 mm to remove large particles, which were watered, covered, and left for approximately seven days 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 24h 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.

2.2. 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 3200 ×*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.

2.3. 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 cyclers (300PE) following the supplier's instructions.

2.4. 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 (Larsen *et al*., 2023; Arnault *et al*., 2022). The RCbray index was estimated using the package *iCAMP* (Ning *et al*., 2020).

3. 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).

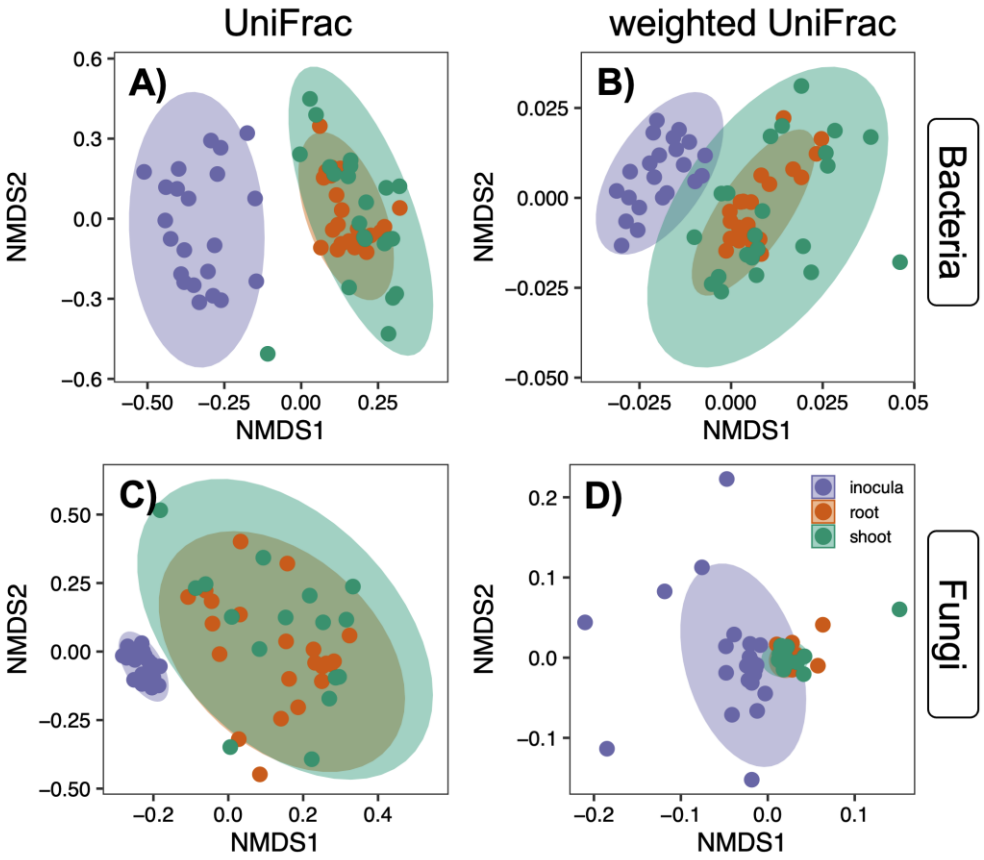

***Figure 1**. NMDS (Non-Metric Multi-Dimensional Scaling) of bacterial (A, B) and fungal (C, D) communities of inocula, root, and shoot samples, using unweighted (A, C) and weighted (B, D) distances between samples. Ellipses represent 95% CI for each compartment.*

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).

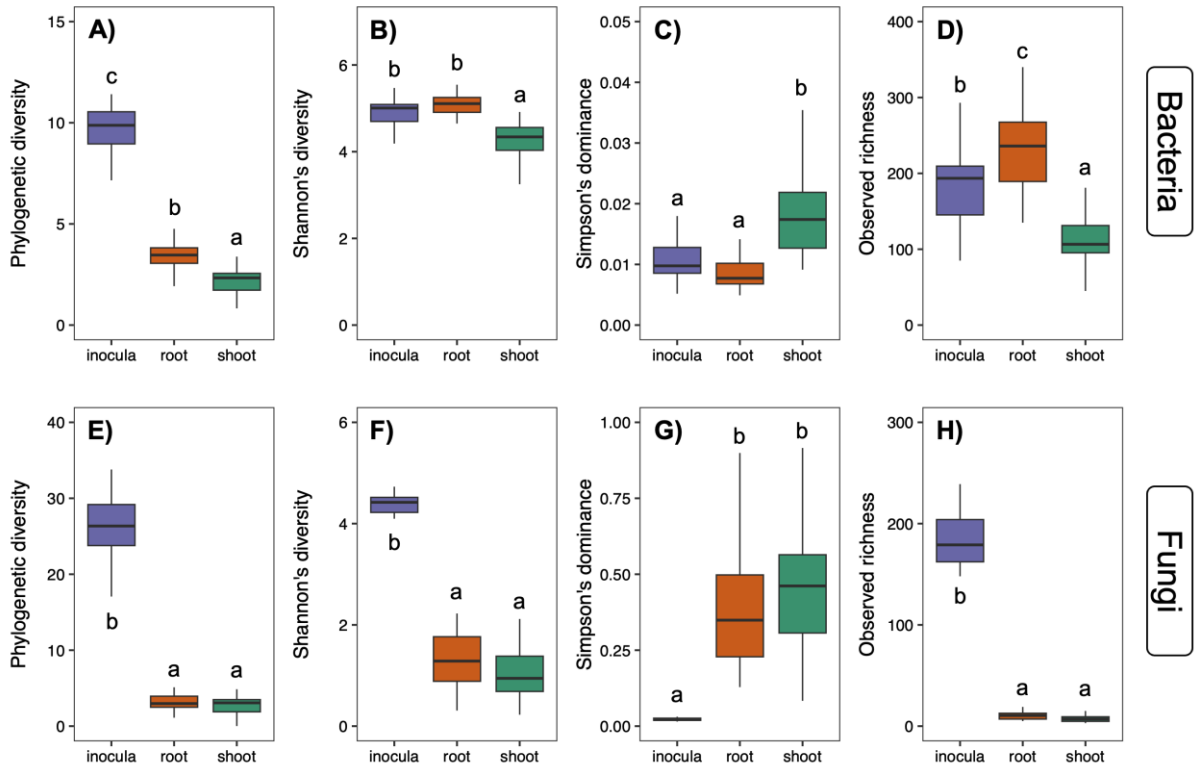

***Figure 2**. Phylogenetic diversity (A and E), Shannon diversity (B and F), Simpson dominance (C and G), and observed richness (D and H) indexes for bacterial (A, B, C, D) and fungal (E, F, G, H) communities in samples collected from inocula, root, and shoot. Pairwise comparisons are shown as letters for each boxplot, and exact p-values are reported in Tab. S1.*

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 3 B). Although 87 bacterial ASVs were enriched in both roots and shoots compared to the inoculum, 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).

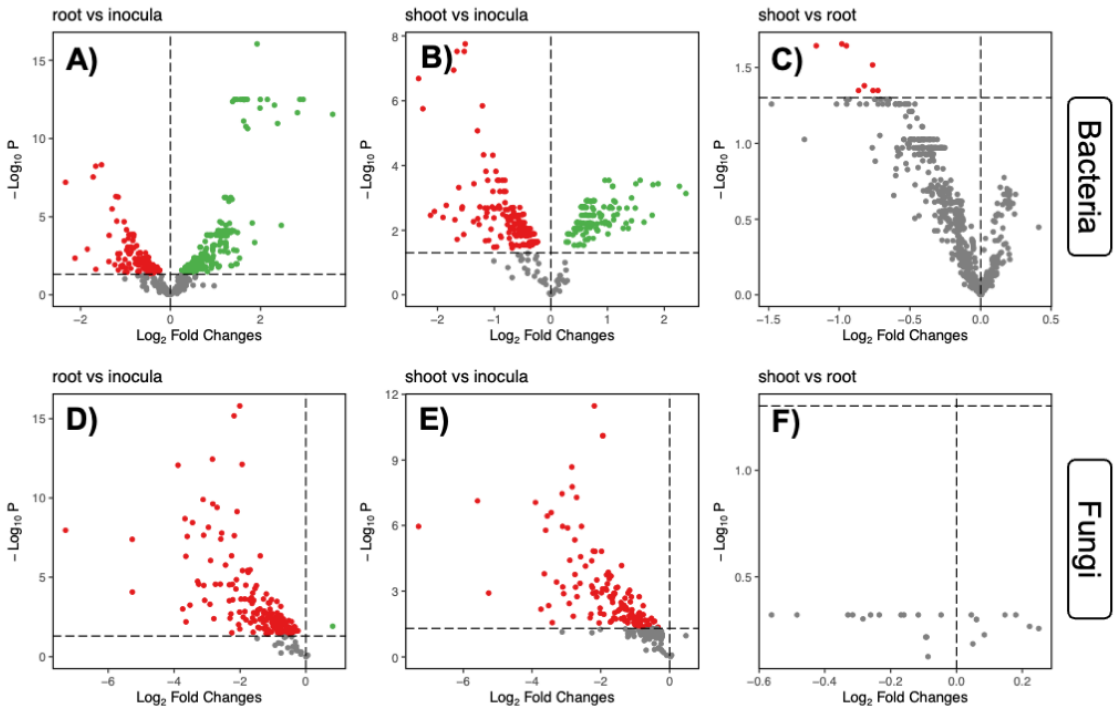

Figure 3*. Volcano plots show differentially abundant ASVs of bacteria (top) and fungi (bottom) between pairs of compartments. (A and D) root (green) vs inocula (red), (B and E) shoot (green) vs inocula (red), (C and F) shoot (green) vs roots (red). ASVs in grey are not differentially abundant between the two compartments.*

Further tests showed that the number of ASVs shared between compartments (Fig. 4A and 4 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).

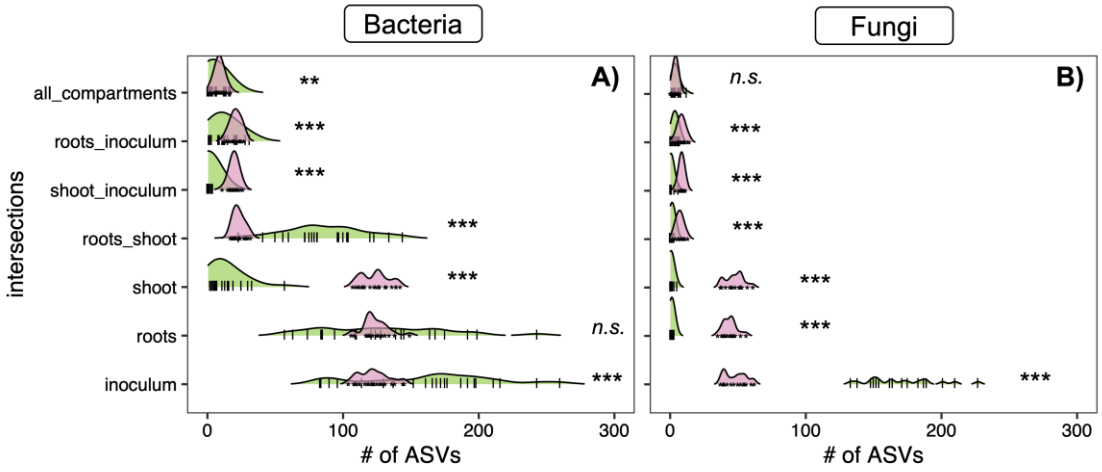

Figure 4*. Ridgeline plot showing the distribution of the number of ASVs shared between compartments and testing the difference between observed data (green distribution, vertical lines are individual datapoints) and randomized data (pink distribution, small stars are individual datapoints). Asterisks 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).*

To further explore the idea that the microbiome of gnotobiotic plants is assembled through deterministic processes rather than stochastic 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 5 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 always > 2 (Fig. 5C and 5D), suggesting that deterministic processes contributed to the assembly of plant microbiomes.

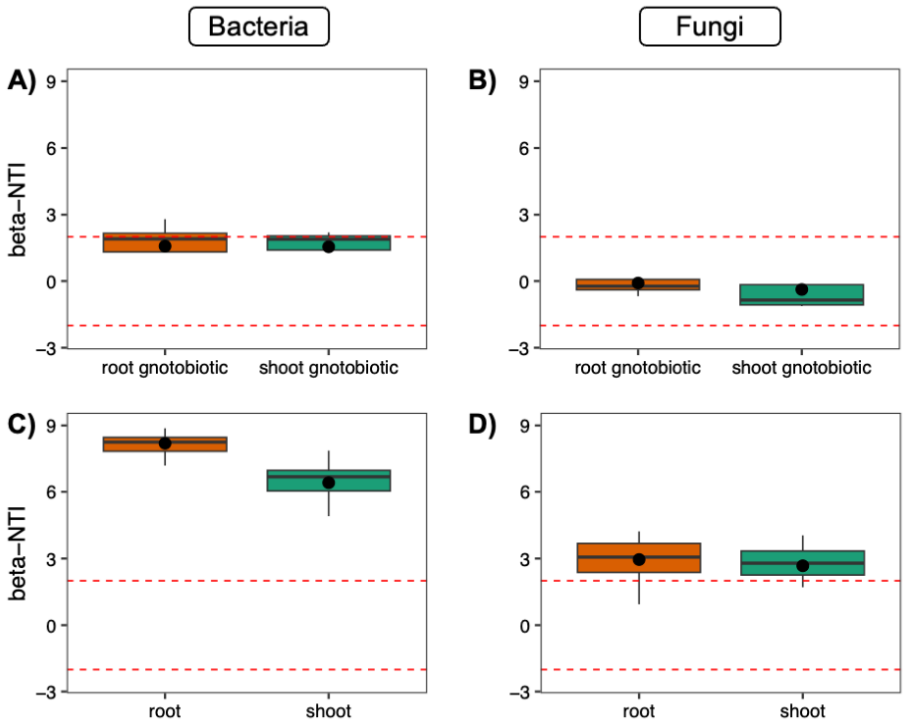

Figure 5. *β-NTI (beta-nearest taxon index) of bacterial (A and C) and fungal (B and D) communities associated with gnotobiotic (A and B) and inoculated (C and D) lettuce plants. Horizontal dashed lines represent reference values of -2 and 2, indicating that the thresholds in the microbiome assembly are considered to be shaped by stochastic processes. The black dot at the top of the boxplot represents the distribution mean.*

4. 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 inoculated plants was different from that of the inoculum, and that the microbial 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. 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 stronger 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 (Chaparro *et al*., 2013; Badri *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 did not observe any 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 nine 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.

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Chapter 3. Experimental selection of a microbial community for postharvest diseases control

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Abstract

The field of plant microbiome research is currently receiving increased attention due to its significant contribution in mitigating postharvest diseases, which have a negative effect on global fruit production. Despite the successful isolation and application of certain biological control agents, their effectiveness and expansion are constrained by various limitations. The aim of this study was to develop a strong and resilient microbial community that can effectively contrast postharvest diseases. This was achieved by subjecting microbial communities obtained from five different plant sources (lichenes, apples, pyrus, oranges, and quinces) to selective pressure over ten re-inoculation cycles using apple fruits as a model. We then used the microbial community from the first and tenth cycles to inoculate apple fruits together with two important postharvest pathogens *Botrytis cinerea* and *Penicillium expansum*. Our findings indicated significant changes in the structure and diversity of the fruit microbiome over the re-inoculation cycles, which led to microbial assemblies able to reduce the incidence and severity of postharvest diseased by 90% (*B. cinerea*) and 60-70% (*P. expansum*).

Keywords: plant microbiome, postharvest disease, amplicon metagenomics

1. Introduction

One of the primary causes of food loss and waste is the postharvest diseases caused by various pathogens. During the postharvest phase, these losses may account for 10 to 50% or more of the total harvest and may have major implications for food safety and security (Guan *et al*., 2023). To mitigate and prevent these losses, a wide range of chemical pesticides or fungicides have been extensively utilized, resulting in a variety of negative consequences such as the emergence of fungicide-resistant strains of pathogens and the accumulation of chemical residues within fruits, potentially endangering human health (Kumari *et al*., 2022; Spadaro and Gullino, 2004). It is crucial to seek a reliable and sustainable alternative to replace harmful agrochemicals like the use of beneficial microbial isolates as biocontrol agents. However, there are several constraints that limit the broad adoption and accessibility of these agents in the market. The limitations encompass inconsistent and unreliable performance under various conditions, a narrow field of activity, and a short shelf life (Usall *et al*., 2016). Another significant limitation is that a substantial proportion of environmental microbes, exceeding 95%, cannot be cultivated. This implies that only a small fraction of the potentially advantageous microorganisms can be cultured and manipulated for agricultural purposes (Qiua *et al*., 2019).

To overcome some of these problems, a new approach is currently gaining scientific attention which is Host-Mediated Microbiome Engineering. This approach involves utilizing whole microbial communities with specific desired traits to establish a stable system that yields lasting beneficial effects. This is achieved through artificial selection and the implementation of a multigeneration strategy (Mueller *et al*., 2021). The objective of this strategy is to enhance the growth and development of the host in accordance with the targeted trait, which may include acquiring resistance against biotic or abiotic stress or experiencing an increase in growth rate (Mueller and Sachs, 2015). When conducting this approach, three factors should be considered: the host plant, a reliable source of microbial community as an inoculum, and a stress pressure to stimulate plant response, which will improve artificial selection (Durán *et al*., 2021; Rodríguez *et al*., 2023). Multiple investigations have been carried out applying this approach, such as the utilization of microbial community derived from Antarctic soil over a span of 10 cycles to establish a stable beneficial community capable of influencing tomato growth in a water-deficient environment (Rodríguez *et al*., 2023). Another study used *Arabidopsis thaliana* and its associated microbial community to select for soil microbiomes inducing earlier or later flowering times of their hosts after 10 cycles (Buisse *et al*., 2015).

This study aimed to harness the microbial community of apple fruits against postharvest disease to create a stable community with long-term beneficial effects. This was achieved through a multi-generational and artificial selection strategy employing epiphytic microbial communities obtained from five distinct sources as an initial inoculum. By subjecting microbiomes to selection pressure through ten cycles of rapid reinoculation, we aimed at identifying a microbial community that is antagonistic to postharvest diseases. Our hypothesis is that the development and growth of the two pathogens will be inhibited by the microbial community selected after several re-inoculation cycles.

2. Materials and methods

2.1. Initial inoculum preparation and first cycles

The initial inocula were prepared from different sources (lichens, apples, pyrus, oranges, and quinces) by gently rubbing a cotton swab on the surface of each source. After that, swabs were transferred to a 15 ml falcon tube containing 10 ml sterile PBS buffer (sodium chloride 8 g/l, potassium chloride 0.2 g/l, sodium phosphate dibasic 1.44 g/l and potassium phosphate monobasic 0.245 g/l). Then, the swabs were rotated several times to release the bacterial cells into the buffer. Next, 80 µL of each inoculum was used for inoculating separate group of apple fruits (a group for each inoculum). A small amount of each inoculum was preserved in 40% glycerol stock, and the rest was stored for DNA extraction and further analysis at -80°C.

Apple fruits underwent a two-minute surface sterilization process utilizing 1% sodium hypochlorite. Subsequently, the apples were dried and labelled with a numerical value ranging from one to sixty, in addition to four marks being made on the apical portion near the stem to indicate the wounds places. Following that, apples were arranged in two boxes randomly in semi-sterile conditions, with 30 apples per box, and four wounds were created in the previously marked area using a sterile needle. Each wound was inoculated with 20 µL of the corresponding inoculum group previously mentioned and PBS buffer was inoculated the same way as a control. Each group including the control had ten apples as replicates. Boxes were supplied with moistened tissue paper as a source of humidity and stored at room temperature.

2.2. Fruit sampling and inoculation preparation for passaging lines

After three days from inoculation, the sampling procedure started by preparing sixty 2 ml Eppendorf tubes, each labeled sequentially from one to sixty, and filled with 1.5 ml of sterile PBS buffer. Then, a sterile scalpel was used to collect the area surrounding the wounds from each apple, which was then transferred to the corresponding Eppendorf tube. Afterwards, apple pieces were crushed using separate sterile pestles (one for each Eppendorf) for releasing the microbial community into the buffer. They were then used as a source of inoculation for the next cycle where 20 µL of each Eppendorf was used to inoculate each wound by taking into consideration the numbering system. In other words, collected samples from the first apple of the first cycle were used to inoculate the first apple from the second cycle and so on. This process was carried out the same for the six groups throughout the successive cycles (from the second to the tenth cycle). The experiment progressed at a rate of two cycles per week, with each cycle spanning a duration of three to four days. Each cycle comprised sixty apples, distributed among six distinct groups: five inoculum, one control, and ten apples for each group. Apple fruits (golden delicious variety) were obtained from a commercial orchard that operates under organic agricultural practices. On a weekly basis, approximately 300 fruits were transported to the laboratory and stored in a refrigerator at a temperature of 4°C until they were utilized.

The number of bacterial colony-forming units (CFUs) was estimated by inoculating the 10^5 dilution of each inoculum onto R2A lab-agar medium and allowing them to incubate at room temperature for 24 hours. This test was carried out for the first, third, fifth, seventh and ninth cycles. Sample preservation process was the same as the initial inoculum where a small amount was

preserved with 40% glycerol and the rest was stored for DNA extraction and further analysis and they were kept at -80°C.

2.3. DNA extraction and library preparation

Samples were centrifuged at max speed (20,700 x*g*) for 15 minutes to obtain a pellet, the supernatant was removed, and the pellet was resuspended in 300 mL of lysis buffer (10 mM Tris, 100 mM NaCl, 20% SDS). After that, the DNA was extracted by using phenol-chloroform method then DNA concentration and quality were estimated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Libraries for amplicon metagenomics were prepared by amplifying a portion of the 16S rRNA gene (V3-V4) by using primers 515f/806r (Caporaso *et al*., 2012). The amplifications were conducted using the standard two-step PCR method. After that, libraries were quantified using a Qubit fluorometer (Thermo Fisher Scientific), pooled together in equal amounts and sequenced on an Illumina MiSeq platform (Illumina, CA, USA) using the MiSeq Reagent Kit v3 600 cyclers (300PE), according to the manufacturer instructions.

2.4. Final test with postharvest pathogens

This experiment aimed at evaluating the antagonistic effect of microbial communities from the first and tenth cycles when co-inoculated with two important apple postharvest pathogens, *Botrytis cinerea* and *Penicillium expansum*. For each cycle, samples collected from the previous six inoculation groups were used (lichenes, apples, pyrus, oranges, quinces and PBS buffer) along with PBS buffer acting as the new control.

This experiment used live microbial communities that were previously collected and conserved using a 40% glycerol solution at -80°C. The replicates from each inoculum group were pooled together, resulting in a total of six inoculation groups per cycle: apples, pears, oranges, quince, lichen, and PBS. A volume of 100 µL was used from each group to inoculate 50 mL of LB broth medium. The medium was then left to grow overnight at 23°C with gentle shaking at 120 rpm. Following that, the pellet obtained from centrifuging the microbial solution was subsequently resuspended in sterile PBS buffer and used for apple wounds inoculation.

Botrytis cinerea and *Penicillium expansum* were both cultivated on PDA medium in five plates at room temperature for a duration of one week. Under sterile conditions, 2 ml of sterile distilled water was added to each plate and thoroughly mixed to gather the conidial cells. The conidial suspensions from all plates were pooled together and then measured five times using the Thoma cell counting chamber to determine the mean concentration. Ultimately, the conidial suspension was adjusted to a final concentration of 10^5 conidia/ml before being used as the pathogen inoculum.

A total of 420 apple fruits were utilized and subjected to the same procedures as before, including surface sterilization, drying, marking with numbers, creating four wounds, and distributed randomly among 14 boxes. For each inoculation group, 30 apples were used as replicates, and each wound was inoculated with 20 µL and then kept for around two hours at room temperature to allow absorption and colonization of the microbial community. After that, replicates from each group were divided as follows: 10 apples were inoculated with the *B. cinerea*, 10 apples were inoculated with the *P. expansum* and the last 10 apples were inoculated with PBS buffer. The pathogen inoculation procedure was the same as the one used to inoculate the microbial community. All

apples were kept inside closed boxes with moistened tissue paper at room temperature for six days or until the first signs of infection appeared. After that, the infection diameter of each wound from each apple was measured over the subsequent four days. Following this, samples were collected from the non-infected wounds and subjected to the previously mentioned sample processing.

2.5. Data analysis

The number of bacterial colony-forming units (CFU)/ml was calculated by multiplying the total count of colonies of each plate by the dilution factor (10^5) and then dividing by the volume of the culture plate. The data underwent a log10 transformation prior to being used for the aim of fitting a linear model, using the *lme4* (Bates *et al*., 2015) and *car* (Fox and Weisberg, 2018) packages. Cycle, inoculum, and their interactions were considered as fixed factors then pairwise post hoc test was computed using the package *emmeans* (corrected using false discovery rate, FDR) (Lenth, 2022).

The final testing with postharvest pathogens required the identification of two parameters: disease incidence, which refers to the presence or absence of symptoms, and disease severity, which measures the degree of symptom expression and the lesion diameter (Michailides *et al*., 2009; Manso and Nunes, 2011). The disease incidence was determined by observing the number of wounds that exhibited symptoms in each apple. As for disease severity, it was computed by multiplying the infection diameter of each wound by four and dividing it by the maximum diameter value for each cycle in the entire dataset. Afterwards, we calculated the disease index by multiplying the pathogen disease severity and incidence, and then converting the result into the natural logarithm. To test these data, we used the same analysis procedure as before by fitting a separate linear model for each pathogen. Cycle, inoculum, time point, and their interactions were considered as fixed factors then pairwise post hoc test was computed.

2.6. Pair end reads and further analysis

The analysis of microbial community data was conducted using R v4.3.2 and data visualization was created using *ggplot2* (Wickham, 2009). The *DADA2* pipeline (Callahan *et al*., 2016) was employed to process the paired end reads, eliminating low-quality data, identifying Amplicon Sequence Variants (ASVs), and removing chimeras. The Taxonomy assignment was performed using SILVA v138 (Quast *et al*., 2013). After that, a *phyloseq* object was created by merging the ASV table, taxonomical annotation for each ASV, and a phylogenetic tree which results in 8864387 reads from 600 samples. The data underwent an initial filtration process where ASVs identified as "chloroplasts" or "mitochondria" (McMurdie and Holmes, 2013) were removed. Subsequently, the package *decontam* (Davis *et al*., 2018) was utilized to eliminate any contaminants that could have resulted from non-template and negative controls which result in 6196152 reads from 551 samples. Ultimately, the ASV table underwent normalization using the *wrench* package (Kumar *et al*., 2018) and was utilized for all analyses, except for the computation of alpha diversity metrics (i.e., phylogenetic diversity, Shannon diversity, and observed diversity).

The Permutational Multivariate Analysis of Variance (PERMANOVA) method was used to test the variation in microbial communities across cycles resulting from different inoculum. The process involved the creation of a distance matrix and specifying cycle, inoculum, and their interactions as fixed factors. The *'adonis'* function from the vegan package in R was utilized to conduct this test,

which relied on weighted UniFrac distances. The outcomes were then displayed using nonmetric multidimensional scaling (NMDS) (Oksanen *et al*., 2007). Subsequently, we conducted additional tests to assess the impact of various cycles on the microbial composition. The package *RVAideMemoire* (Hervé, 2022) was used to make pairwise contrasts, with p-values adjusted for multiple comparisons applying the false discovery rate (FDR) correction.

Faith's phylogenetic diversity, Shannon's diversity, and observed diversity were used to infer the alpha diversity of microbial communities in each sample and across cycles. This was accomplished using two packages: *picante* package phylogenetic diversity calculations (Kembel *et al*., 2010) and *microbiome* package for Shannon's and observed diversity calculations (Lahti and Sudarshan, 2019). The test was conducted by fitting individual linear mixed effects models for each index, using the *lme4* (Bates *et al*., 2015) and *car* (Fox and Weisberg, 2018) packages. Cycle, inoculum, and their interactions were considered as fixed factors, while block (= experimental box) were treated as random factors. A pairwise post hoc test was computed using the package *emmeans* (corrected using false discovery rate, FDR) (Lenth, 2022).

In order to determine if the microbial assembly process is controlled by deterministic or stochastic processes, we compute the Beta Nearest Taxon Index (βNTI) using a null model with 999 randomizations using the package *picante* (Larsen *et al*., 2023; Arnault *et al*., 2022). We then define $|\beta NTI| \geq 2$ as the dominant deterministic process and $|\beta NTI| < 2$ as the dominant stochastic process (Xiong *et al*., 2021).

3. Results

First, we evaluated the changes in the microbial community structure within six inoculum groups and across multiple cycles. The microbiome structure was influenced by inoculation cycle ($F_{9,493} = 11.25$, $p < 0.001$), the inoculum group ($F_{5,493} = 107$, $p < 0.001$), and their interaction ($F_{43,493} = 2.66$, $p < 0.001$) (Fig. 1). The inoculum group accounted for a greater proportion of the variance (43%) in comparison to inoculation cycle (8.1%). A post hoc test revealed a significant variation in the structure of microbial communities among the six inoculum groups ($p = 0.001$ for all pairwise comparisons, FDR-corrected). Focusing on single cycles, we found that the variation explained by inoculum group was higher in the first five cycles (ranging from ~60%), and thereafter decreased gradually towards the tenth cycle (~40%) (Fig. S1).

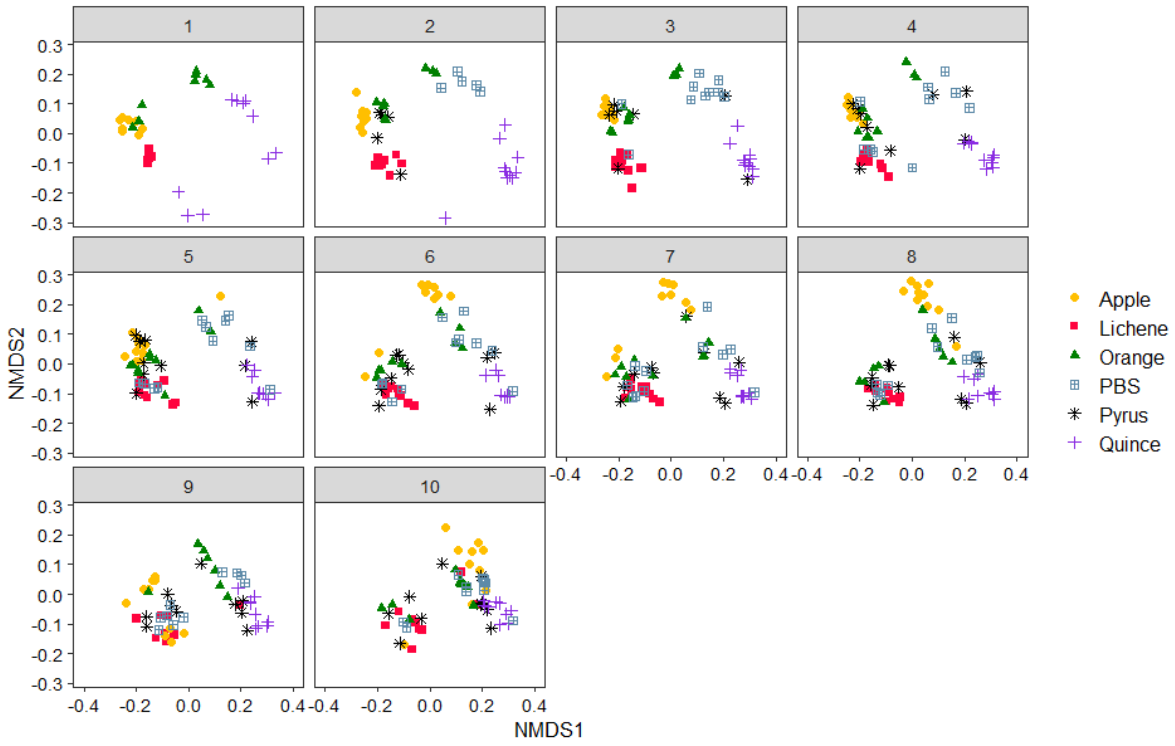

Figure 1*. NMDS (Non-Metric Multi-Dimensional Scaling) of microbial communities of six inoculum groups (Apple, Lichene, Orange, Pyrus, Quince and PBS) across ten cycles.*

We also compared the number of CFU across multiple cycles. We found that the number of CFU were different among cycles ($F_{4,270} = 37.77$, $p < 0.001$), inoculum groups ($F_{5,270} = 12.53$, $p < 0.001$) and their interaction ($F_{20,270} = 10.12$, $p < 0.001$). A post-hoc test showed that the first cycle in all inoculum groups had a lower number of CFU when compared to the rest of the cycles. Furthermore, the data indicated that there was no significant difference in CFU count among the third, fifth, seventh, and ninth cycles in most of the inoculum groups (Fig. 2).

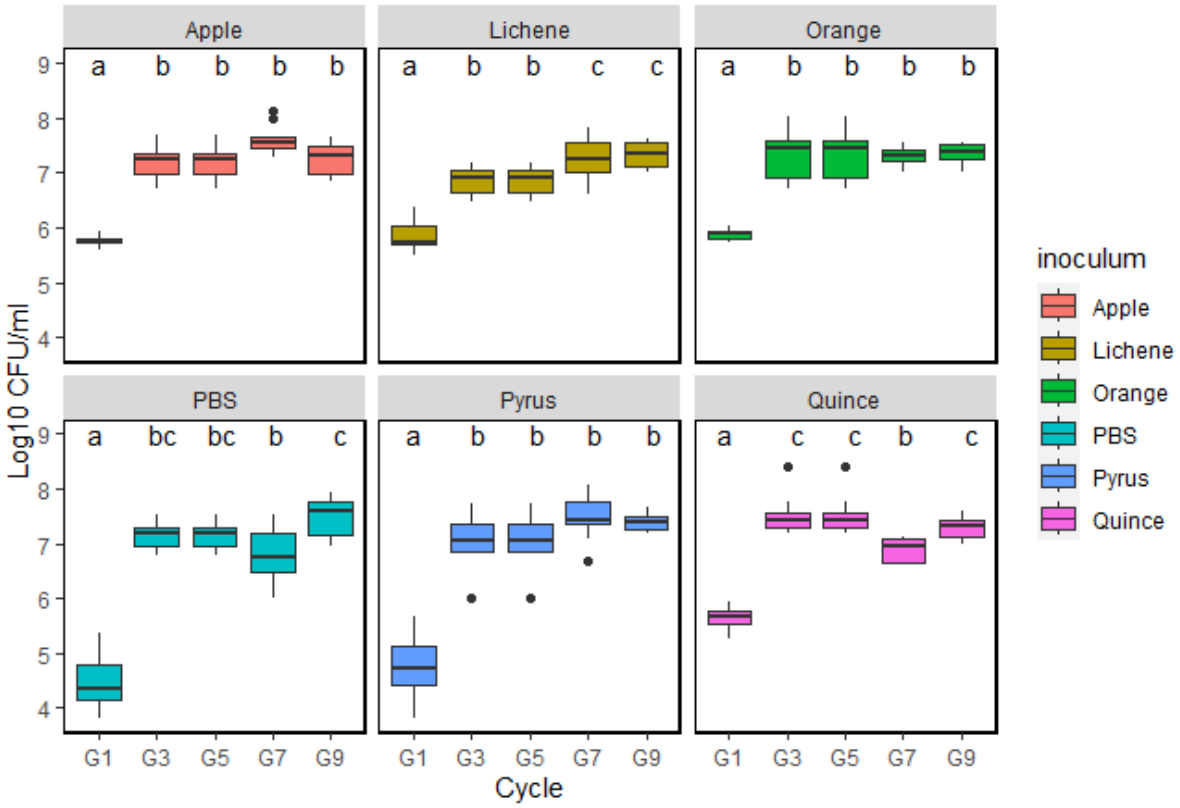

Figure 2. *Number of bacterial colony-forming units (CFU)/ml reported in log10 for six inoculum groups across first, third, fifth, seventh and ninth cycles.*

We also found that the cycle, inoculum, and their interaction had an impact on the richness (observed diversity) and abundance (Shannon diversity) of microbial communities (Table. 1, Fig. 3). Post hoc contrasts showed significant rise in both the observed species richness and Shannon diversity in the lichen, Pyrus, as well as in the PBS treatment starting from the second cycle. Regarding the orange and quince inoculum, there was no significant difference observed among the different cycles. While in the apple inoculum, the richness and abundance exhibited an initial increase over the first eight cycles, followed by a slight decrease in the last two cycles. On the other hand, whereas phylogenetic diversity was significantly impacted by the inoculum group ($p < 0.001$), there was no significant difference observed within each inoculum throughout the cycles (Fig. 3).

Table 1*. Results from testing the effect of multiple cycles, six inoculum groups and their interaction on the phylogenetic, Shannon and observed diversity.*

		Phylogenetic diversity		Shanonn diversity		Observed diversity	
	df	Chisq	*P*	Chisq	*P*	Chisq	*P*
Cycles	9	34.636	<0.001	197	<0.001	113.86	<0.001
Inoculum	5	85.924	<0.001	169.59	<0.001	187.54	<0.001
cycles: inoculum	43	129.023	<0.001	154.27	<0.001	119.94	<0.001

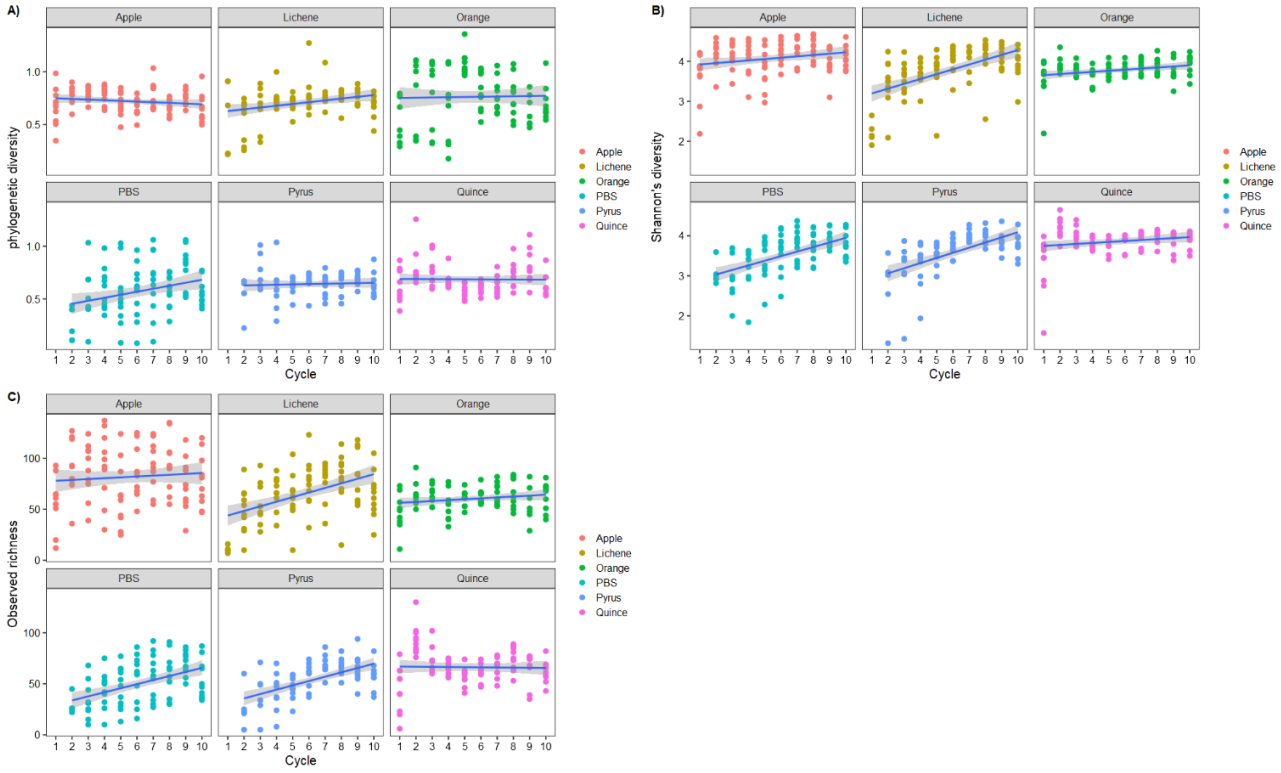

Figure 3*. Faith's phylogenetic diversity (A), Shannon's diversity (B), and observed diversity (C) indexes for microbial communities collected from six different inoculum groups and across ten cycles.*

Further analyses revealed that the interplay of deterministic (|βNTI|≥ 2) and stochastic (|βNTI|< 2) processes in the assembly of the apple microbiome across different inoculum groups was significantly influenced by the re-inoculation cycles. During the initial five cycles, we observed a heightened contribution of stochastic processes (βNTI between -2 and 2) (Fig. 4). This contribution gradually diminished with the progression of cycles, eventually shifting towards deterministic (βNTI > 2) in the last four cycles (Fig. 4).

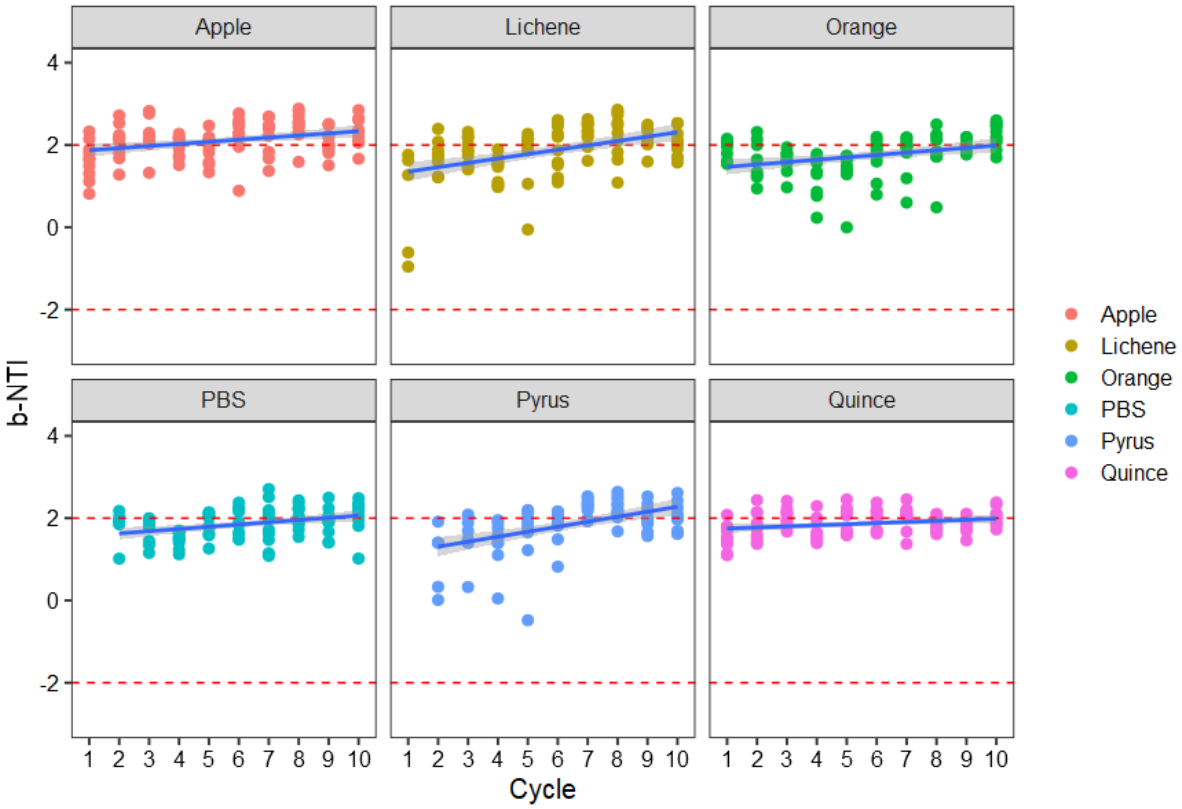

Figure 4 *βNTI (beta- Nearest Taxon Index) of microbial communities from six different inoculum groups across ten cycles. The reference values of -2 and 2, are shown by the horizontal dashed lines.*

The microbial communities of the first and tenth cycles were co-inoculated with two important postharvest pathogens. The results indicated that cycles and time points had a significant influence on the disease index. Regarding *B. cinerea*, the cycle factor contributed to explain a larger portion of the variance (42.19%) compared to the time point factor, which accounted for 27% of the variance (Fig. 5A, Table. S1). On the other hand, in *P. expansum*, the time point explained the largest proportion of variance (40%) while inoculum explained the 26% (Fig. 5B, Table. S1). Furthermore, none of the apples that were co-inoculated with PBS buffer and the inoculum derived from the two cycles showed any signs of rot. The post hoc contrast test revealed that both cycle and time point had an impact on the disease index in both pathogens. Specifically, greater values were seen in the first cycle ($p < 0.001$) when compared to the control and from the second time point to the end of the experiment ($p < 0.001$) across all inoculum groups.

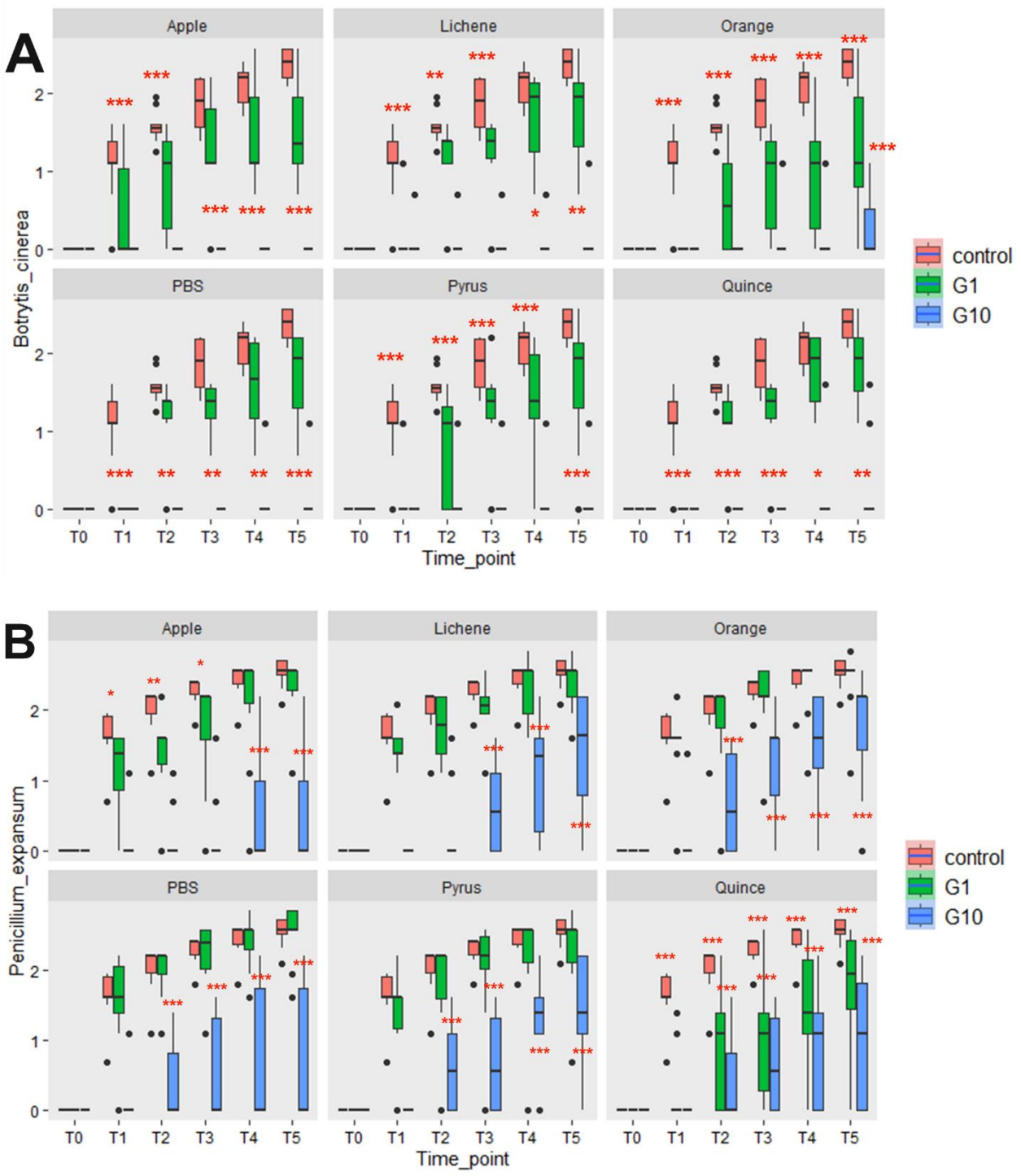

***Figure 5**. Disease index of Botrytis cinerea (A) and Penicillium expansum (B) after 5 (T0), 6 (T1), 7 (T2), 8 (T3), 9 (T4) and 10 (T5) days of inoculation. Significant differences between control and each cycle are reported with an asterisk symbol.*

4. Discussion

To the best of our knowledge, this study is the first to use a microbiome artificial selection strategy to select and create a stable microbial community for the purpose of preventing postharvest diseases. Over the course of ten cycles, a response to the selection, most apparent after the second and the third cycles, was evident in both the gradually increase in richness, abundance and number of calculated CFU of the microbial communities in most of the inoculum groups. The

microbiome selection process significantly influenced the selection of beneficial traits, resulting in a noticeable decrease in postharvest rot by two main postharvest pathogens, *B. cinerea* and *P. expansum*.

During the initial cycles of the experiment, we observed a gradual rise in the richness and abundance of microbial species, which then plateaued between the eighth and tenth cycles in microbial communities collected from apple, lichene and pyrus groups. On the other hand, the microbial communities collected from orange and quince did not change in the richness and abundance over the course of ten cycles. This was evident from the number of observed ASVs, Shannon diversity, and number of microbial CFU. Previous study has demonstrated an opposite pattern, revealing a decrease in the richness of microbial species during the initial cycle (Morella *et al*., 2020; Jochum *et al*., 2019). In both previous studies, microbial communities were obtained from soil, which is characterized by more complex and diverse microbial communities. Microbial communities collected from the surface of five different plant sources were used in our experiment. These communities are not as complex as those obtained from soil and when combined with our selective pressure, this may have favored the assembly of fast-colonizing microorganisms. Furthermore, our findings align with those of Shankar *et al*., (2023), who found that, when eight cycles are utilized, the microbial community's richness tends to stabilize in the final cycles. One possible explanation for the persistence of alpha diversity metrics across cycles is that members of the microbiome community have evolved to withstand strong selection pressures, which has improved host fitness (Gruber-Dorninger *et al*., 2015; Nottingham *et al*., 2022). More fit microbes eventually outcompete less fit ones in the host environment (Nottingham *et al*., 2022; Zegeye *et al*., 2019; Shankar *et al*., 2023).

The results of our study also indicated that the βNTI values in earlier cycles varied between -2 and 2. This implies the occurrence of a stochastic process, potentially triggered by the introduction and migration of new microbial communities within the apple fruits. The assembly process was altered in the later cycles where βNTI values exceeded 2. This suggests an increasing influence of selective pressure (deterministic processes) such as plant genotype over multiple cycles of ecosystem selection which was observed in previous studies (Shankar et al., 2023; Morella et al., 2020). This pattern was observed in all groups of inoculums. Regarding the PBS, our re-inoculation process involved introducing microbial communities obtained from the previous cycle (specifically, apple endophytes). This demonstrated that even when re-inoculating with similar microbial communities, there is still dispersal and a dynamic exchange of microbial populations in the first cycles (stochastic process).

Manipulating the epiphytic fruit microbiome has demonstrated potential in the biological management of postharvest pathogens. Hammami *et al*. (2022) discovered that five distinct bacterial strains obtained from citrus fruit peels had the ability to manage green and blue mold in citrus fruits. In their study, Manso and Nunes (2011) found that *Rahnella aquatilis*, a yeast strain isolated from the surface of apples, effectively decreased the occurrence of the pathogen *Penicillium expansum* on apple fruits by 78%. The utilization of microbial isolates, either individually or in synthetic communities, presents a straightforward method to specifically target particular pathogens. However, this approach is constrained by various factors, such as the interaction with

the original microbial community and the limited specificity towards only one or two pathogens. We aimed to establish a resilient microbial community that would have lasting positive impacts on postharvest pathogen. We used the complete epiphytic microbial community obtained from five plant sources as an initial inoculum for a multigenerational experiment, in which we applied selection pressure. This approach demonstrated its ability to selectively cultivate a microbiome that promotes favorable growth and ultimately yields a final cycle with advantageous traits, such as enhancing plant resilience to water scarcity (Rodríguez *et al*., 2023) or increasing plant biomass (Shankar *et al*., 2023). These results might be due to the selection and colonization of rare and unculturable taxa that might influence plant development and tolerance against biotic and abiotic factors.

Similarly, our findings demonstrated that inoculation with microbial community coming from the last cycle reduced the disease index of *B. cinerea* to nearly zero emphasizing the potential disease-suppressive nature of the microbiomes. This effect was observed in all inoculum groups. Using the same microbial community against *P. expansum* yielded the same results only at the first time point. However, the disease index of *P. expansum* was notably decreased in apple fruits that were inoculated with the tenth cycle, particularly the microbial communities of apples, PBS, and quince (with reductions of 78%, 70%, and 60% in the pathogen disease index after 10 days of inoculation, respectively), in comparison to the control. The antagonistic effect of the microbial community obtained from the PBS group against both pathogens could be attributed to the enrichment of beneficial microbiome agents from apple endophytes, and further research is needed to understand the mechanism underlying this effect. The limited impact of the microbial communities from the tenth cycle on inhibiting the growth of *P. expansum* may be attributed to the fact that this pathogen is significantly more aggressive compared to *B. cinerea*, especially apples and pears (Louw and Korsten, 2014).

In conclusion, this study demonstrates the successful use of a microbiome engineering strategy to select and create a stable microbial community that can reduce the incidence of postharvest diseases in apple fruits. The composition of microbial communities varied over cycles, with a shift from stochastic to deterministic processes in the final cycles. Manipulating the microbiome of fruits shows promise in the management of postharvest pathogens, but further research is needed to understand the selection process, application techniques and functional traits of the microbial communities collected from the final cycle.

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Chapter 4. Global warming alters plant-microbiome interaction with consequence on plant fitness

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Abstract

Given the escalating challenges of climate change, understanding how plants adapt to increasing temperatures, and how this influences the interactions between plants and their microbiome, is of paramount importance. This study investigates the impact of increased environmental temperature on the interaction between the plant *Spirodela polyrhiza* and its microbiome, and the subsequent effects on plant fitness. Using a bacterial synthetic community composed of 18 bacterial isolates, we inoculated 99 different genotypes of *S. polyrhiza* under two temperature conditions. Our findings suggest that temperature, genotype group, and sampling time point significantly influence the growth and development of *S. polyrhiza* genotypes, and this effect was modulated by the plant microbiome. Our results shed light on the relationships between plant microbiomes and their responses to changing temperatures, providing valuable insights into the interplay between plant phenotypic plasticity, microbial communities, and climate change.

Keywords: microbial synthetic community, phenotypic plasticity, *Spirodela polyrhiza*

1. Introduction

Global warming has caused an unexpected rise in temperature, posing a serious threat to global agricultural production (Ahmad *et al*., 2022). Additionally, it causes a significant shift in our climate which is accompanied by an increase in the frequency and severity of heat waves, droughts, and events that cause stress on plants. As a result of increased cumulative CO_2 emissions over the last century, the average global surface temperature increased by 1.09 °C between 2011 and 2020 compared to 1850 and 1900, and it is expected to rise again to 1.5 °C by 2040 (IPCC Climate Change, 2023). A slight temperature increase has the potential to be stressful and negatively impact plant growth and development in general. This could result in a decrease in biomass, flowering and fruiting, and, ultimately, significant yield losses (Ahmad *et al*., 2022). Understanding and addressing the challenges posed by climate change is critical not only for plant production but also for meeting global food demand.

Plants have evolved a range of stress response mechanisms such as alterations in their physical structure, biological functions, and growth patterns, that allow them to cope with the challenges of adapting and surviving in the presence of climate change (Ashra and Nair, 2022). Phenotypic plasticity is an important adaptive strategy that enables a genotype to express multiple phenotypes in response to different environmental conditions, thereby increasing their ability to withstand the impacts of any change in the surrounding environment (Matesanz *et al*., 2010). The development of this adaptive trait is generally determined by two main factors which are genetics and environmental factors (Ledón-Rettig and Ragsdale, 2021). Under high temperature conditions, plants respond by increasing the number and thickness of their leaves (Banowetz *et al*., 2008). They also gradually accumulate soluble sugars (Sasaki *et al*., 1996), proteins, and chlorophyll content (Liu *et al*., 2011) as a means to adapt to these adverse conditions (Nievola *et al*., 2017). Gaining a comprehensive understanding of how plants utilize phenotypic plasticity is essential in the context of a shifting climate. In addition, several studies have shown that introducing growth-promoting bacteria like *Pseudomonas* sp. and *Bacillus* sp. to plants can have a positive impact on their growth under heat stress conditions (Munir *et al*., 2022; Kang *et al*., 2019). Thus, the plant microbiome has the potential to modulate plant phenotypic plasticity, allowing the host to adapt to new environmental conditions. For example, Malacrinò et al. (2024) found that both herbivory-induced responses and changes in the plant microbiome alter the genotype frequencies within plant populations, and this in turn increases the resistance to further herbivory. However, the role of temperature-mediated interactions between plants and their microbiomes remains unexplored, particularly in how these interactions may influence plant adaptation and resilience under global warming scenarios.

This work aimed to fill this gab and examine the impact of an increase in environment temperature on the interaction between plants and their microbiomes, and the consequences of these changes on plant fitness by using *Spirodela polyrhiza* as a model species. A bacterial synthetic community composed of 18 bacterial isolates was used to inoculate 99 different genotypes of *S. polyrhiza* under two temperatures. After six and twelve days of inoculation, three measurements were taken (number of fronds, surface area, and dry biomass). First, we investigated whether increasing the temperature during cultivation of different duckweed genotypes affected their

growth. We hypothesize that growing under these conditions will increase the number and surface area of fronds in specific genotypes. Second, we investigate the effect of inoculation with a bacterial synthetic community across different genotypes and temperatures, assuming that their interaction will influence shifts in plant growth and development.

2. Material and method

2.1. Temperature regimes

In accordance with the findings of Bastin *et al.*, (2019) and the global worming scenario, it is anticipated that by 2050, more than 77% of the world's most populous cities will be impacted by global warming. It is also expected that 22% will experience a climate that no major city is presently exposed to. Considering this, we have selected Cologne and Hamburg, two major cities in Germany, and their projected climate conditions, which are anticipated to resemble the current climate in San Marino, Italy. The temperatures of these two major cities (Cologne and San Marino) were specifically selected for the month of July using a comprehensive online dataset that includes 30 years of hourly weather model simulations and observations (Meteoblue). These observations provide a reliable representation of climate patterns across the whole year. The daytime temperature in Cologne and Hamburg (***A***) is 23°C, while the nighttime temperature is 13°C. San Marino (***B***) experiences a daytime temperature of 27°C and a nighttime temperature of 13°C. For the purpose of this study, the temperature in Cologne is regarded as the control variable.

2.2. Duckweed genotypes and experimental overview

Spirodela polyrhiza stock collection were obtained from Professor Xu's lab (iomE Institute, JG University of Mainz, Mainz, Germany). The collection was preserved in germ-free conditions at 18°C with a day/night period of 12:12 in Erlenmeyer flask filled with full N medium (KH2PO4 150 µM, KNO3 8 mM, Ca(NO3)2 1 mM, H3BO3 5 µM, MnCl2 13 µM, Na2MoO4 0.4 µM, MgSO4 1 mM, FeNaEDTA 25 µM) (Appenroth, 2023). A total of 99 genotypes from different clonal families and populations were utilized from this collection. We used 4, 56, 8, and 31 genotypes from the populations of America, Asia, Europe, and India, respectively (list in S1 Table). Prior to this experiment, 10-12 fronds of each sterile genotype were transferred to 250 ml Erlenmeyer flask filled with 150 ml of 1:10 full N medium for a pre-cultivation period of three weeks. During the pre-cultivation period, plants were grown in climatic chambers with an irradiance of 130-140 mol/m^2/s, a photoperiod of 16 h/8 h day/night and two temperature conditions, Cologne (***A***: 23°C day and 13°C night), San Marino (***B***: 27°C day and 13°C night). After 11 days of pre-cultivation, the medium was changed to a new one to refresh and renew the lost nutrients.

On the inoculation day, 7 fronds (= individual plants) were transferred under semi-sterile conditions to sterile plastic beakers (PP, transparent, round, 250 ml, Plastikbecher.de GmbH) containing 100 ml of fresh 1:10 full N medium with bacterial synthetic community (see below) in three biological replicates for each temperature (***A*** and ***B***). The plastic beakers were closed with a sterile perforated lid (PP, transparent, round, 101 mm, Plastikbecher.de GmbH) to prevent water condensation and allow air circulation inside the beaker. Following inoculation, pictures were taken for each beaker to measure frond surface area at T0. The beakers were periodically rearranged and

shuffled within the growth chamber every three to four days due to the uneven distribution of light intensity across the entire bench.

Due to the fast growth rate of *S. polyrhiza* and the potential for bacterial cells to undergo changes within a few hours, the total number of genotypes was divided into six batches, with each batch consisting of 14 to 23 genotypes. For every batch, a newly prepared inoculum was created, and a small amount was stored in a 50% glycerol solution for DNA extraction and further analysis. Consequently, two genotypes (SP046 and SP063) were chosen randomly and introduced in every batch to monitor and stabilize the entire system (in total 109 genotypes). Controls were introduced in the last batch for 21 genotypes by inoculating with heat-killed bacterial community in addition to the normal inoculum.

2.3. Synthetic microbial community construction

The synthetic microbial community (SynComm) was built from eighteen bacterial isolates. These bacteria were isolated from outdoor duckweed fronds in the summer of 2020 and stored in 60% glycerol stock at -80°C. Preparation of the SynComm was according to Ishizawa *et al*., 2020 and Bodenhausen *et al*., 2014 with few modifications. Briefly, each isolate was stroked on LB agar and incubated at 28°C for approximately 24 hours. Subsequently, one loop of the bacterial colony was inoculated into 150 ml of LB liquid medium and allowing it to grow overnight at 28°C with gentle shaking (130 rpm). Afterwards, the bacterial cells were collected via centrifugation at 10,000×g for 8 minutes at 4 °C. The supernatant was then removed, and the cells were re-suspended in sterile 1:10 full N medium. In the end, all isolates were mixed at the same cell densities determined by the optical density at 600 nm (OD600), to reach final OD of 0.4. To ensure homogeneity, the final inoculum was shaken for thirty minutes at 175 rpm then mixed at 10 % rate with fresh 1:10 full N medium and mixed well before using as a growth substance. For the control inoculation in the last batch, a new bacterial community was prepared as described above and autoclaved for 1 hour at 121°C to kill the bacterial cells.

2.4. Harvesting

Samples were collected at two time points, six (T1) and twelve (T2) days after the introduction of the bacterial inoculum, to monitor the changes in the microbial community across different generations. During each time point, the number of fronds, surface area, and dry biomass were measured. Additionally, ten fronds were collected from each replicate, flash frozen using liquid nitrogen, and then freeze-dried prior to DNA extraction procedures.

2.5. Statistical analysis

All data were analyzed in R v4.3.2 and data visualization was created using *ggplot2* (Wickham, 2009). Dry biomass, frond surface area and frond reproduction rate were calculated for each genotype at each time point as phenotypic traits. Frond reproduction rate was calculated by this formula: $(LnFNt6 - LnFNt0)/(t6 - t0)$, where FN is the number of fronds calculated at each time point (Ziegler *et al*., 2015). For each sample, the surface area was determined by capturing an image of the fronds, which also included a floating reference square, and processed using the R package *pliman* (Olivoto, 2022). The final data was split into two groups: one consisting only inoculated genotypes, and the other consisting of both inoculated and control genotypes from

the sixth batch. Linear mixed effects models were used to investigate differences in the three parameters across genotypes and temperatures. Temperature, population, time points, and their interactions were treated as fixed factors, while genotypes were treated as random variables. In the second data set, the same model and factors were used, along with treatment as a fixed factor. A pairwise post hoc test was computed using the package *emmeans* (corrected using false discovery rate, FDR) (Lenth, 2022).

3. Results

This analysis was conducted using two different data sets. The first data set did not include any controls and was used to examine the interaction between microbial inoculation, temperature fluctuations, and their impact on plant fitness. First, we focused on the change in fronds reproduction rate which was influenced by temperature (F = 960.68; df = 1; $p < 0.001$), population (F = 27.74; df = 3; $p < 0.001$), time point (F = 1879.63; df = 1; $p < 0.001$) and the interaction effect of population with temperature and time point separately (Fig. S1). Since the interaction between temperature and time points did not change significantly, it is possible that the effect of temperature on the reproduction rate was constant throughout all time points. The reproduction rates of genotypes belonging to three populations, America, Asia, and India, increased at higher temperatures, whereas there was no significant variance in the Europe population. Using a post hoc test, we examined the various genotypes at both temperatures at the second time point (12 days) in pairwise comparison and found that genotype 29 ($p = 0.0001$), 36 ($p = 0.001$), and 20 ($p = 0.01$) had significantly different frond yields per day according to the temperature regime (Table S2).

Second, the change in frond surface area was influence by temperature (F = 8.54; df = 1; $p = 0.001$), population (F = 69.65; df = 3; $p < 0.001$), time point (F = 503; df = 2; $p < 0.001$) and the interaction between temperature and time point (F = 50.39; df = 2; $p < 0.001$) (Fig. S2). A post hoc test showed a significant change in fronds surface area of six genotypes (belonging to Asia population) across the different temperatures ($p < 0.001$, = 0.001) (Table S2). Lastly, temperature (F = 43.73; df = 1; p 0.001), population (F = 42.50; df = 3; p 0.001), time point (F = 849.48; df = 1; p 0.001), and the interaction between temperature and population as well as population and time point all had an effect on the change in fronds dry biomass (Fig. S3). The post hoc test showed a difference between time points across all population where an increase in fronds dry biomass observed in the second time point.

The same analysis was performed on the second data set, which included both inoculated and non-inoculated genotypes, to disentangle the effects of temperature and microbial inoculum on plant growth parameters. Results indicate that temperature and time point factors contribute to a higher portion of variance in fronds reproduction rates (28% and 19%, respectively) compared to treatment and population. In all cases, the reproductive rate was higher at higher temperatures, but the magnitude of this effect varied when plants were exposed to the synthetic microbial community (Fig. 1).

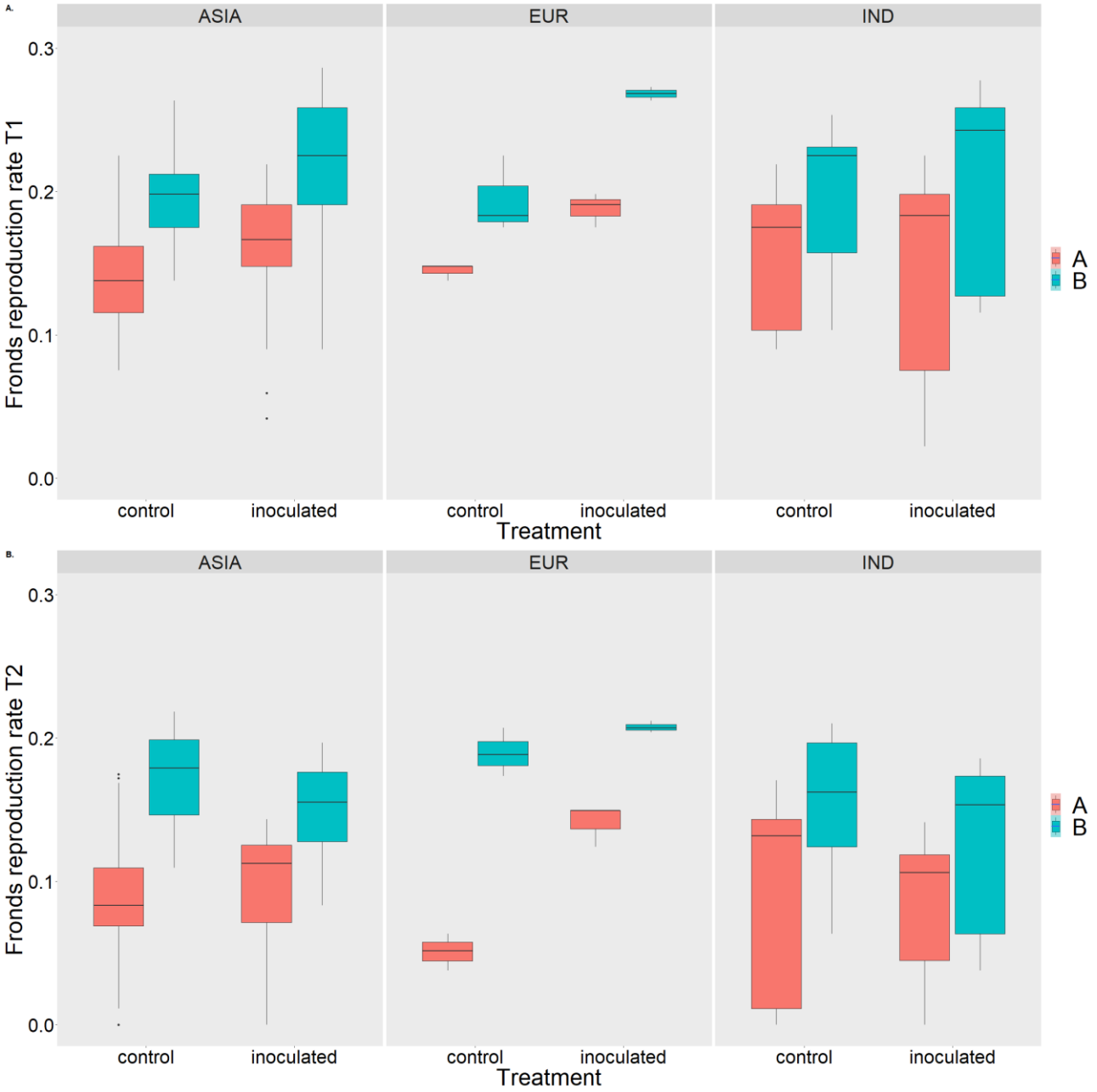

Figure 1. Fronds reproduction rates across four populations (America, Asia, Europe and India) after 6 (T1) and 12 (T2) days of live or heat-killed bacterial inoculation (inoculated and control, respectively). Two different temperatures were used Cologne (A, 23°C day, 13°C night) and San Marino (B, 27°C day, 13°C night)

Moreover, both population (15%) and time point (26%) had a significant impact on changes in frond surface area. After six days of inoculation, the surface area per frond of both inoculated and non-inoculated plants exhibited similar patterns across different population, with higher values under the influence of higher temperature in Asia and Europe populations. Regarding the second time point, surface area of non-inoculated plants was higher at higher temperatures. As for the Indian population, it expressed the highest values in the non-inoculated plants under both temperatures (Fig. 2).

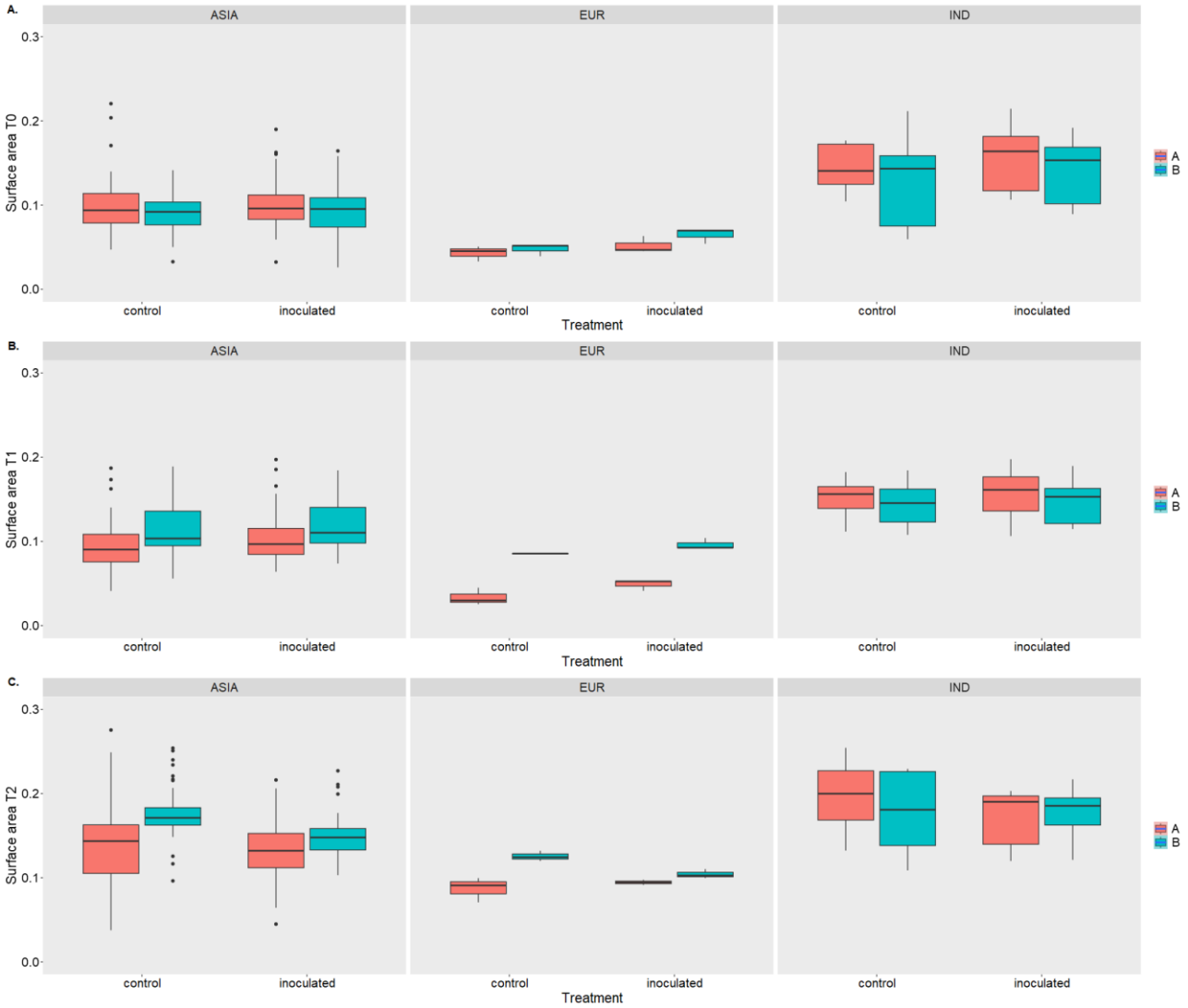

Figure 2. Fronds surface area across four populations (America, Asia, Europe and India) after 6 (T1) and 12 (T2) days of live or heat-killed bacterial inoculation (inoculated and control, respectively). Two different temperatures were used Cologne (A, 23°C day, 13°C night) and San Marino (B, 27°C day, 13°C night)

Similar to the surface area, population (18%) and time points (19%) contributed to a larger portion of the variance when compared to the other factors. After twelve days of inoculation, India population showed higher dry biomass values when compared to the other two populations especially in the inoculated plants at control temperature.

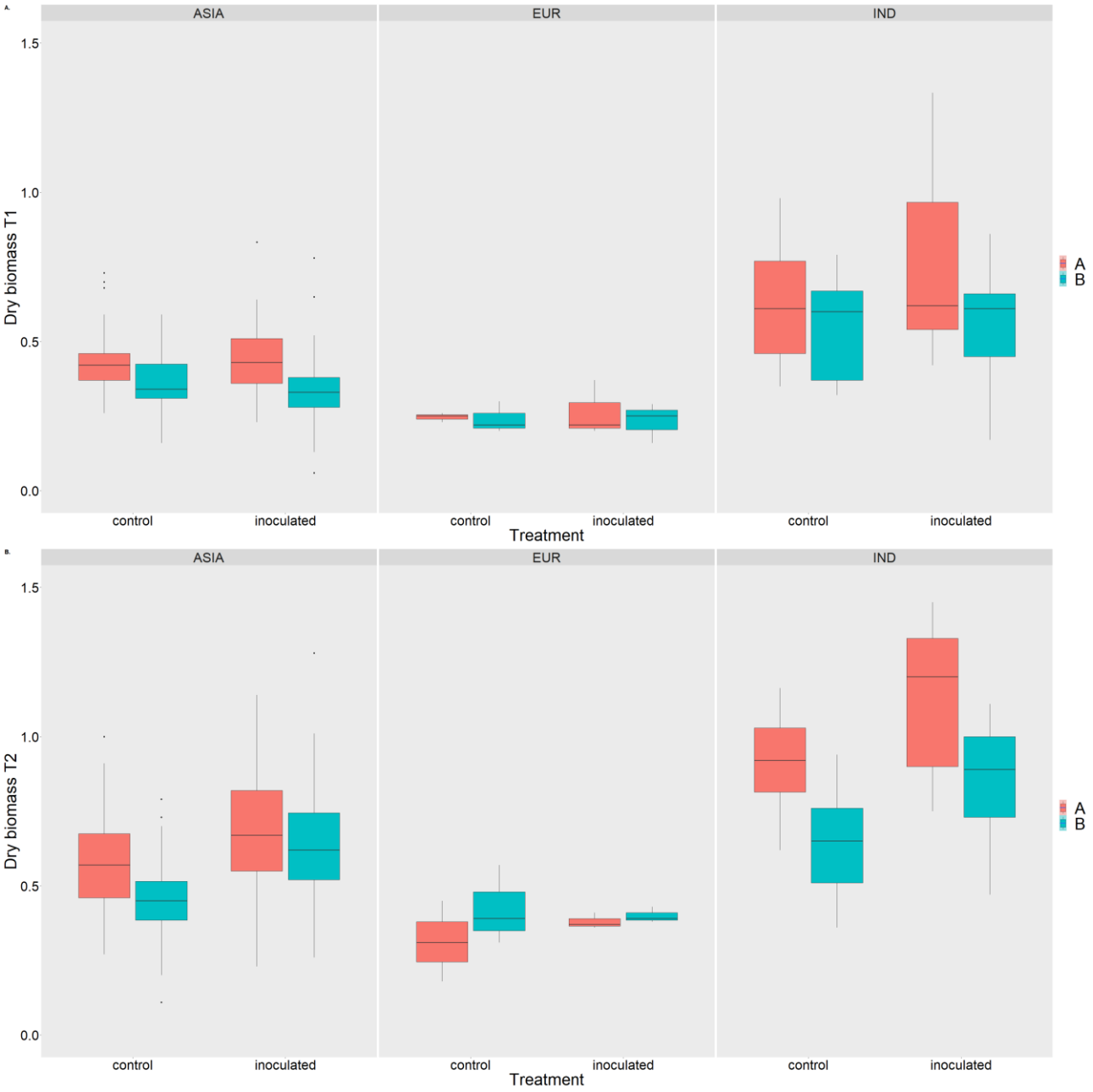

Figure 3. Fronds dry biomass across four populations (America, Asia, Europe and India) after 6 (T1) and 12 (T2) days of live or heat-killed bacterial inoculation (inoculated and control, respectively). Two different temperatures were used Cologne (A, 23°C day, 13°C night) and San Marino (B, 27°C day, 13°C night)

4. Discussion

This experiment involved cultivating multiple genotypes of *S. polyrhiza* in the presence of either a live bacterial microbial community or a heat-killed community, while subjecting them to varying temperatures. The aim was to investigate and separate the combined impact of the microbial community and temperature on the plant's ability to exhibit phenotypic plasticity. Our findings demonstrated that temperature, population, and time point significantly impacted changes in phenotypic traits such as surface area, dry biomass, and fronds reproduction rates. After twelve days of inoculation, we noticed that each population responded differently. For example, under control temperature, the Asia population showed an increase in both the number of fronds and dry

biomass. The Europe population also showed an increase in reproduction rate along with a reduction in fronds size and dry biomass. These changes might be due to the combined effect driven by the bacterial SynComm and the different temperature regimes. O'Brien et al. (2020) found that co-inoculating duckweed plants with microbial communities showed a positive effect on plant phenotypic traits under zinc stress, whereas Schäfer and Xu (2022) found the opposite to be true for herbivory stress.

When looking at the influence of our treatments (temperature and inoculation) we found an interesting effect on the number of fronds produced by each group of genotypes. Overall, plants grown under higher temperatures produced a higher number of fronds, and often this effect was heightened by the presence of a microbial community. This shows that increases in the temperature can have rapid and profound effects on the structure of plant populations and, thus, of plant communities, as the fitness of the different plant genotypes is influenced by both the host intrinsic phenotypic plasticity, but also by the microbiome-induced phenotypic plasticity. Indeed, we also found that the morphology of duckweed fronds was altered by the combined effect of temperature regimes and the plant-associated microbial community. Higher temperatures often resulted in larger fronds with a lower biomass, but the magnitude of these changes was significantly influenced by the microbial community.

Overall, our results support the idea that, in the short term, plant adaptation to global changes will be mainly driven by their interaction with the microbial community (Trivedi et al., 2022). Raising temperature influences both the host and its microbiome. Understanding these interactions allows us to predict how plants will adapt to future climate scenarios by focusing on the importance of microbial communities in mediating plant responses to temperature changes, thereby informing strategies for enhancing plant resilience in the face of global warming. While the host will respond through changes to its phenotype induced by the novel abiotic conditions, also the microbiome will be affected by changes in the environment. In turn, changes in microbiome composition and/or function can have profound impact on the plant biology, and thus on its phenotype. Our results support this hypothesis, and further analyses will help in disentangling the mechanisms of these complex relationships, and to better understand their consequences on plant ecology and evolution. It is indeed pivotal to rapidly push this field further, as hacking the plant-microbiome interactions under new challenging climates will be essential to guarantee the future of agricultural production and the preservation of natural ecosystems.

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Chapter 5. Conclusions and Future Perspectives

Mohamed N.Z., Li Destri Nicosia M.G.

The combination of climate change and the increasing global population presents substantial risks to agricultural productivity. The rising need for food, along with the negative impacts of climate changes such as heatwaves, droughts, floods, and the spread of plant diseases, require the investigation of sustainable approaches to improve plant growth. A promising strategy involves leveraging the abilities of plant microbiomes. These microbial communities, comprising bacteria, fungi, and other species, have the ability to improve plant resistance and promote growth through several mechanisms. Gaining insight into the complex structure of these microbiomes can pave the way for the creation of sustainable agricultural systems that can adapt to changing environmental conditions, ensuring long-term food security and ecological balance.

First, a systematic review was preformed to identify common technical difficulties discussed in studies published in 2021 that focused on the complex field of plant microbiome. This investigation revealed a wide range of approaches used to analyze the architecture of microbial communities. This involved a variety of methods for extracting DNA, using different sets of primers that target both bacteria and fungi, and employing multiple sequencing technology. The present diversity in these methods caused a substantial obstacle to the standardization of operations within the plant microbiome field. An important finding from this analysis was that 171 of the examined papers lacked raw data. The absence of transparency and publicly available data limits the capacity to compare and reproduce these studies and perform meta-analyses. This research highlighted the substantial impact of standardizing DNA extraction and primer pairings on the results of amplicon metagenomics analyses, despite the inherent difficulties involved in doing so. It is crucial to acknowledge and tackle these problems to ensure the quality and comparability of the data generated in future investigations.

Although there is an increasing interest in the field of microbial research and the recent advancements made in this area, there are still numerous unanswered problems regarding microbial assembly, their interactions with host plants in response to changing environmental conditions, and much more. In this study, three models were created using gnotobiotic approaches to clarify the mechanisms behind plant microbiome interaction as it was proven to influence the plant microbiome interactions studies (Molina et al., 2021; Emmenegger et al., 2023). In the first model, gnotobiotic lettuce seedlings were used to investigate the mechanism that govern plant microbiome assembly. Although several studies have explained these mechanisms, the majority of them have focused on plants grown in the field. While this ensures that the results are applicable in real-life conditions, these observations may be susceptible to external factors that influence the plant microbiome's assembly processes (Wang et al., 2023; Xiong et al., 2021). By growing gnotobiotic plants under complete control conditions we were able to eliminate all possible interference factors coming from the surrounding environment. Based on the findings of this experiment, a deterministic process governs the rapid assembly of soil microbes into seedling roots and shoots. Furtherer analysis confirmed that root microbiomes had a greater variety of microbes

than shoot microbiomes. This finding supports the idea that seedlings establish a selective environment beneath the soil's surface, and that through selection from the soil microbial community, they assemble both root and shoot microbiomes. These results represent valuable information to expand our understanding of plant microbiome assembly process especially at early developmental stages. Furthermore, these insights could be pivotal in developing sustainable agriculture strategies that harness the power of plant microbiomes towards plant health and development.

Afterwards, the second study aimed to examine the feasibility of employing host-mediated microbiome engineering and selective pressure to establish a resilient community with persisting advantageous effects against postharvest disease. This approach has been proven to effectively select and create microbial communities with beneficial traits that can enhance plant resilience to water scarcity (Rodríguez et al., 2023) or increase plant biomass (Shankar et al., 2023). This aim was accomplished by using apple fruits as a model system that was inoculated with five different microbial communities across the course of multiple re-inoculations cycles. The results of this study demonstrate that the application of selective pressure on microbial communities significantly influenced the selection and colonization of beneficial microbial agents inside apple fruits resulting in a noticeable decrease in pathogen rot. This was observed in the final test after the co-inoculation processes of the microbial communities resulted from the last cycle with the two pathogens, *Botrytis cinerea* and *Penicillium expansum* with a 90% and 60-70% reduction in disease index, respectively. The findings of this study hold significant importance for sustainable agriculture approaches, as they provide a novel strategy to enhance the resilience of crops and reduce postharvest disease, thereby improving agricultural productivity and sustainability.

Lastly, the third study examined the effects of global warming and temperature changes on plant microbiome interactions and their impact on plant fitness. Gnotobiotic *Spirodela polyrhiza* plants were chosen as our model system due to its unique characteristics. This small, floating plant reproduces asexually and has a rapid generation time of 5-6 days, making it a suitable model for investigating the impact of environmental shifts on plant-microbiome dynamics over several generations. The findings of our study indicate that variations in temperature, population, and time point have a significant influence on alterations in various phenotypic characteristics, and that these effects are mediated by the plant-microbiome interactions within each genotype. The results from this study are pivotal for understanding the effect of global changes in the evolution of plant populations and the effects that might derive on the interactions within the wider ecological community.

In terms of future studies, it is essential to carry out comprehensive investigations to confirm and expand the knowledge gained from our current research activities. For the lettuce experiment (chapter 2), a detailed analysis of the functional traits of the assembled microbial communities from both the roots and shoots will offer insights into their unique roles and contributions. In the context of the apple experiment (chapter 3), it is vital to investigate deeply into the selection process, particularly to understand how it selects beneficial agents. As for the duckweed experiment (chapter 4), it is crucial to investigate the genetic information of each genotype to find genes that are responsible for altering plant phenotypic characteristics in response to global warming conditions.

Identifying these genes and manipulating them could provide a means to counteract the effects of climate change.

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Appendix

1. Supplementary Material: chapter 2

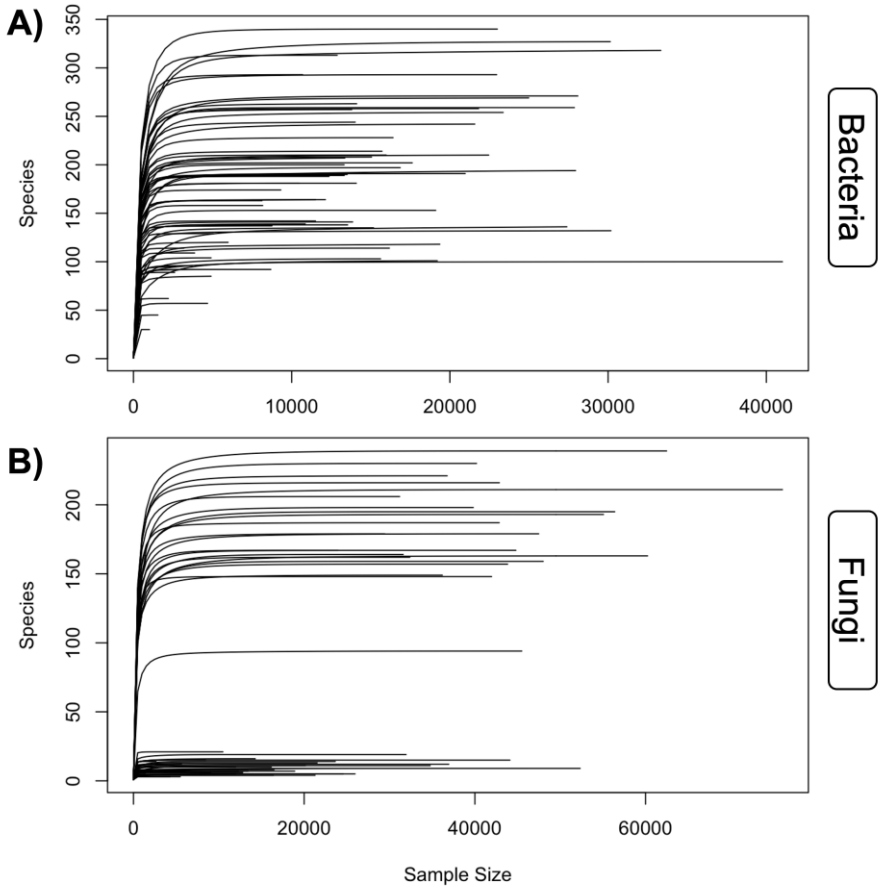

***Figure S1**. Rarefaction curves for (**A**) 16S and (**B**) ITS datasets after cleanup and removal of plastidial reads.*

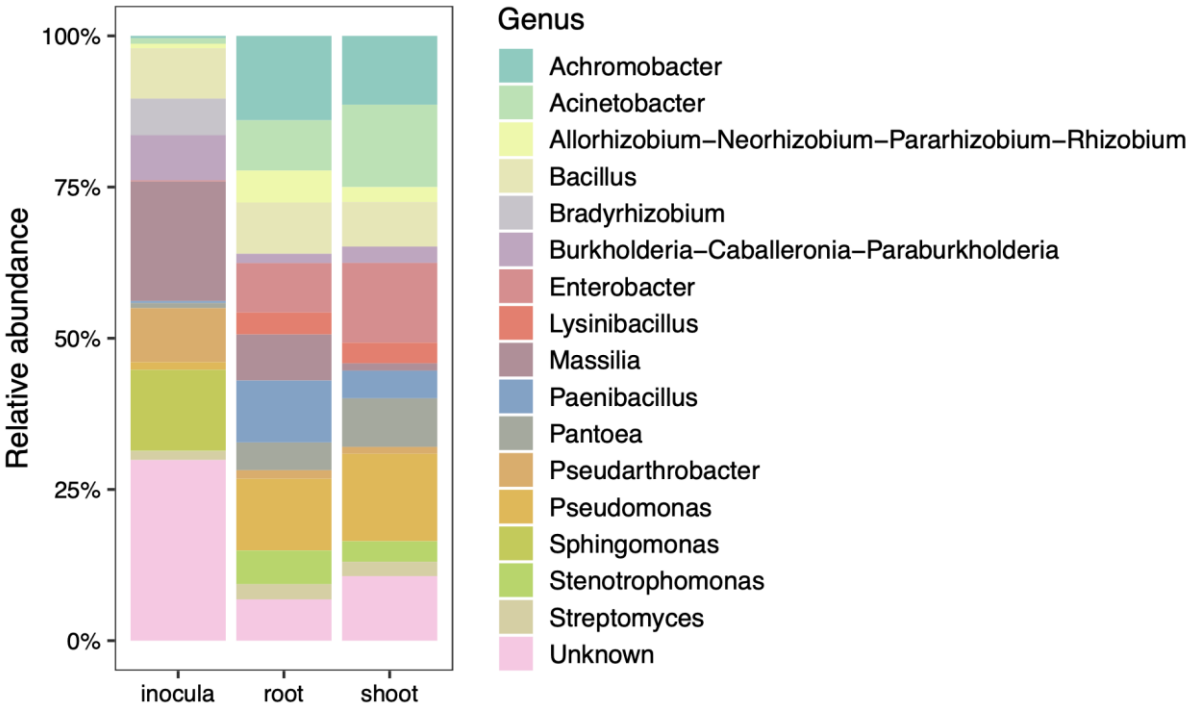

***Figure S2**. Relative abundance (%) of bacterial taxa in inoculated lettuce plants.*

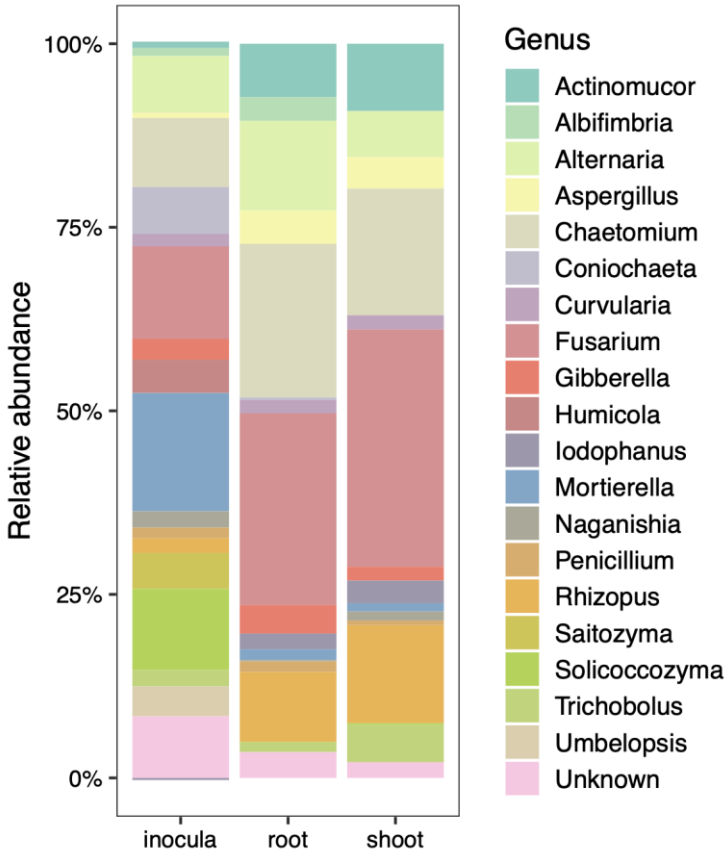

***Figure S3**. Relative abundance (%) of fungal taxa in inoculated lettuce plants.*

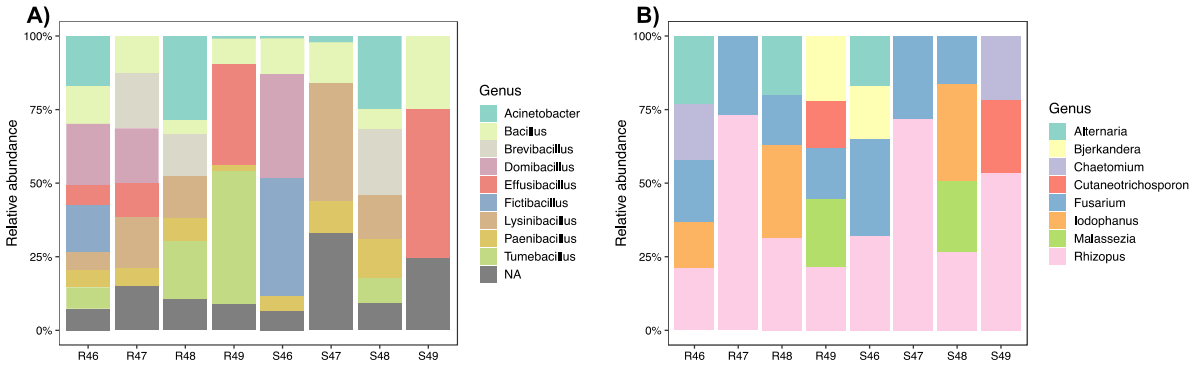

***Figure S4**. Relative abundance (%) of (**A**) bacterial and (**B**) fungal taxa in gnotobiotic lettuce plants. Samples starting with the letter "R" indicate root samples, while those starting with the letter "S" indicate shoot samples.*

***Table S1**. Pairwise comparisons between compartments for different diversity indices, for both bacterial and fungal communities.*

Community	Diversity index	Comparison	p-value (FDR)
Bacteria	Phylogenetic diversity	inocula – root	<0.001
		inocula – shoot	<0.001
		root – shoot	<0.001
	Shannon's diversity	inocula – root	0.167
		inocula – shoot	<0.001
		root – shoot	<0.001
	Simpson's dominance	inocula – root	0.362
		inocula – shoot	0.017
		root – shoot	<0.001
	Observed richness	inocula – root	0.004
		inocula – shoot	<0.001
		root – shoot	<0.001
Fungi	Phylogenetic diversity	inocula – root	<0.001
		inocula – shoot	<0.001
		root – shoot	0.815
	Shannon's diversity	inocula – root	<0.001
		inocula – shoot	<0.001
		root – shoot	0.512
	Simpson's dominance	inocula – root	<0.001
		inocula – shoot	<0.001
		root – shoot	0.440
	Observed richness	inocula – root	<0.001
		inocula – shoot	<0.001
		root – shoot	0.927

2. Supplementary Material: chapter 3

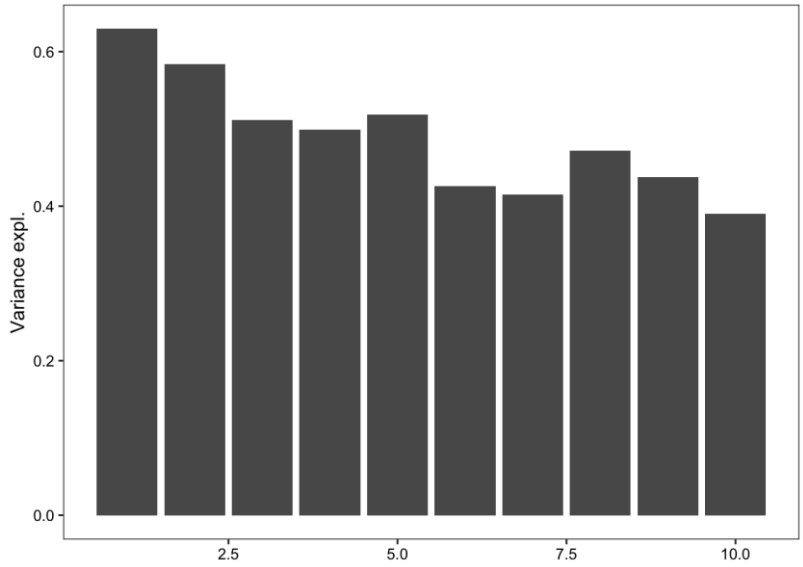

Figure S1. Results of PERMANOVA test showing the microbial communities' structure across cycles.

Table S1. Linear model results from testing the effect of six inoculum groups, two cycles and six time points on disease index of B. cinerea and P. expansum.

		Disease index *(Botrytis cinerea)*			Disease index (*Penicillium expansum)*		
	df	SumSq	F	*P*	SumSq	F	*P*
Inoculum	5	1.37	1.87	-	19.46	14.45	<0.001
Cycle	2	379.10	1303.7	<0.001	316	586.84	<0.001
Time point	5	248.92	342.43	<0.001	479.94	356.5	<0.001
Cycle: inoculum	10	4.40	3.02	<0.001	25.69	9.54	<0.001
Inoculum: Time point	25	1.88	0.51	NA	6.46	0.95	NA
Cycle: Time point	10	117.38	80.73	<0.001	64.36	23.90	<0.001
Cycle: Time point: inoculum	50	4.09	0.56	NA	12.16	0.90	NA
Residuals	972	141.31			281.69		

3. Supplementary Material: chapter 4

Table S1. List of used genotypes, their colonial families and populations.

Genotype Number	Genotype Name	Population	clonal families	Batch number
1	SP017	IND	36	1
2	SP019	IND	38	1
3	SP025	ASIA	41	1
4	SP038	ASIA	45	1
5	SP043	IND	47	1
6	SP046	IND	48	1
7	SP051	IND	50	1
8	SP050	ASIA	49	1
9	SP053	IND	51	1
10	SP056	EUR	52	1
11	SP057	ASIA	53	1
12	SP063	ASIA	54	1
13	SP035	ASIA	44	1
14	SP024	ASIA	40	1
15	SP214	EUR	133	2
16	SP217	EUR	135	2
17	SP220	IND	138	2
18	SP221	IND	139	2
19	SP222	IND	140	2
20	SP223	IND	141	2
21	SP224	IND	142	2

22	SP225	IND	143	2
23	SP226	IND	144	2
24	SP227	IND	145	2
25	SP046	IND	48	2
26	SP063	ASIA	54	2
27	SP229	IND	147	2
28	SP230	IND	148	2
29	SP012	IND	34	2
30	SP234	IND	152	2
31	SP235	IND	153	2
32	SP236	IND	154	2
33	SP237	IND	155	2
34	SP238	IND	156	2
35	SP046	IND	48	3
36	SP063	ASIA	54	3
37	SP048	IND	17	3
38	SP165	ASIA	95	3
39	SP168	ASIA	97	3
40	SP177	IND	101	3
41	SP179	ASIA	103	3
42	SP182	ASIA	105	3
43	SP183	ASIA	106	3
44	SP184	ASIA	107	3
45	SP208	ASIA	128	3

46	SP185	ASIA	108	3
47	SP197	ASIA	117	3
48	SP198	ASIA	118	3
49	SP063	ASIA	54	4
50	SP046	IND	48	4
51	SP126	ASIA	23	4
52	SP117	ASIA	68	4
53	SP114	ASIA	66	4
54	SP127	ASIA	70	4
55	SP145	ASIA	81	4
56	SP142	ASIA	79	4
57	SP155	ASIA	86	4
58	SP150	ASIA	83	4
59	SP153	ASIA	85	4
60	SP148	ASIA	82	4
61	SP157	ASIA	88	4
62	SP054	EUR	10	4
63	SP143	ASIA	80	4
64	SP140	ASIA	77	4
65	SP138	ASIA	76	4
66	SP151	ASIA	84	4
67	SP092	IND	60	5
68	SP063	ASIA	54	5
69	SP085	AME	59	5

70	SP039	IND	16	5
71	SP112	ASIA	64	5
72	SP067	ASIA	19	5
73	SP116	ASIA	21	5
74	SP094	IND	61	5
75	SP083	ASIA	15	5
76	SP074	IND	57	5
77	SP132	ASIA	6	5
78	SP100	ASIA	20	5
79	SP101	ASIA	62	5
80	SP046	IND	48	5
81	SP082	IND	58	5
82	SP014	EUR	7	5
83	SP065	EUR	55	5
84	SP008	AME	9	5
85	SP004	AME	5	5
86	SP011	AME	11	5
87	SP199	ASIA	119	6
88	SP046	IND	48	6
89	SP187	ASIA	110	6
90	SP204	ASIA	124	6
91	SP216	ASIA	134	6
92	SP213	ASIA	132	6
93	SP178	ASIA	102	6

94	SP205	ASIA	125	6
95	SP167	ASIA	96	6
96	SP172	ASIA	100	6
97	SP209	ASIA	129	6
98	SP233	IND	151	6
99	SP240	IND	157	6
100	SP049	EUR	14	6
101	SP161	ASIA	92	6
102	SP201	ASIA	121	6
103	SP063	ASIA	54	6
104	SP210	ASIA	130	6
105	SP186	ASIA	109	6
106	SP202	ASIA	122	6
107	SP119	ASIA	22	6
108	SP188	ASIA	111	6
109	SP037	EUR	13	6

Table S2. Results of post hoc pairwise comparison of each genotype across two temperatures

Genotype	Population	Fronds reproduction rate		Surface area		Dry biomass	
		P value	**Asterisk**	**P value**	**Asterisk**	**P value**	**Asterisk**
SP017	IND	0.102443834		0.668796025		0.830884263	
SP019	IND	0.000163146	***	0.883059091		0.045125456	*
SP025	ASIA	0.028737799	*	0.221190565		0.33354941	
SP038	ASIA	0.000377599	***	0.082756444		0.023119667	*
SP043	IND	0.00779897	**	0.730704765		0.74017903	
SP046	IND	3.98387E-06	***	0.4158544		0.892140909	
SP051	IND	0.005486189	**	0.889159019		0.092708104	
SP050	ASIA	0.051347239		0.824885477		0.250384635	
SP053	IND	0.028215799	*	0.831548677		0.899638595	
SP056	EUR	0.069916264		0.999020012		0.782754268	
SP057	ASIA	0.00165582	**	0.069608778		0.584569968	
SP063	ASIA	0.000680803	***	0.011760979	*	0.000126499	***
SP035	ASIA	0.000969838	***	0.525693409		0.368810108	
SP024	ASIA	0.016537495	*	0.000557828	***	0.690056294	
SP214	EUR	2.40298E-05	***	0.080624106		0.054103075	
SP217	EUR	0.00203481	**	0.139457932		0.076774842	
SP220	IND	0.000840588	***	0.296739408		0.372315097	
SP221	IND	0.000319862	***	0.856590072		0.684485581	
SP222	IND	0.00223318	**	0.218078139		0.73709692	
SP223	IND	0.001601722	**	0.928112902		0.093707308	
SP224	IND	0.001096004	**	0.027164147	*	0.000620098	***
SP225	IND	0.006210795	**	0.448633143		0.28789108	
SP226	IND	0.086613184		0.862595276		0.163430272	
SP227	IND	0.072064541		0.380246323		0.825900332	
SP229	IND	0.050056522		0.77471062		0.881620616	
SP230	IND	0.058119157		0.280107706		0.493032822	
SP012	IND	0.117359924		0.177611631		0.338227819	
SP234	IND	0.002526641	**	0.143007212		0.457086423	

SP235	IND	0.007792544	**	0.005190285	**	0.990629696	
SP236	IND	0.033699886	*	0.877658046		0.873006805	
SP237	IND	0.001228943	**	0.705406649		0.460282503	
SP238	IND	0.004225889	**	0.653921115		0.668910882	
SP048	IND	0.035853018	*	0.887584613		0.00103175	**
SP165	ASIA	0.040710796	*	0.822548414		0.85139932	
SP168	ASIA	0.247295459		0.000524328	***	0.259072679	
SP177	IND	0.000713929	***	0.789489767		0.295516723	
SP179	ASIA	0.007289703	**	0.098902356		0.209201367	
SP182	ASIA	0.001498334	**	0.686563616		0.282499392	
SP183	ASIA	0.000439741	***	0.956687527		0.007619414	**
SP184	ASIA	0.016496743	*	0.533085189		0.908316096	
SP208	ASIA	0.000274657	***	0.000349803	***	0.054618878	
SP185	ASIA	0.003810268	**	0.003221812	**	0.979367998	
SP197	ASIA	0.000193316	***	0.592537945		0.218071384	
SP198	ASIA	0.014042072	*	0.214144088		0.034190207	*
SP126	ASIA	0.017249432	*	0.479470347		0.738484513	
SP117	ASIA	7.60079E-05	***	0.29844878		0.750476841	
SP114	ASIA	0.573444819		0.078686653		0.444664705	
SP127	ASIA	0.014190642	*	0.143711468		0.663491902	
SP145	ASIA	0.030948932	*	0.403321937		0.810443458	
SP142	ASIA	0.028949344	*	0.409867424		0.495559542	
SP155	ASIA	0.003562688	**	0.077535937		0.385114154	
SP150	ASIA	0.017290238	*	0.249654893		0.560053739	
SP153	ASIA	0.000990866	***	0.10770541		0.867014203	
SP148	ASIA	0.000493271	***	0.327113127		0.517417385	
SP157	ASIA	0.002461203	**	0.666283646		0.280559663	
SP054	EUR	0.016368281	*	0.865187696		0.064676894	
SP143	ASIA	0.000314095	***	0.929713616		0.304463238	
SP140	ASIA	0.000788278	***	0.073494578		0.935756408	
SP138	ASIA	0.004701779	**	0.143013288		0.118506835	

SP151	ASIA	0.000143005	***	0.052922963		0.037144841	*
SP092	IND	0.00102657	**	0.016974302	*	0.023193666	*
SP085	AME	0.041619688	*	0.022049473	*	0.032593681	*
SP039	IND	0.002951609	**	0.025462476	*	0.107783107	
SP112	ASIA	0.002456785	**	0.542994569		0.093644166	
SP067	ASIA	0.069450706		0.003202143	**	0.260257496	
SP116	ASIA	0.000155532	***	0.179002972		0.216566011	
SP094	IND	0.004342998	**	0.193398151		0.123158684	
SP083	ASIA	0.000341972	***	0.250276647		0.796283172	
SP074	IND	0.005608481	**	0.191207248		0.405646569	
SP132	ASIA	0.002371487	**	0.203877562		0.143410534	
SP100	ASIA	8.01876E-05	***	0.842970311		0.163559932	
SP101	ASIA	0.010587559	*	0.265718307		0.930305866	
SP082	IND	0.001509802	**	0.942498643		0.060129609	
SP014	EUR	0.001780044	**	0.543793677		0.920823941	
SP065	EUR	0.000436343	***	0.013353988	*	0.020835429	*
SP008	AME	0.000965019	***	0.077851727		0.711248272	
SP004	AME	0.012904854	*	0.109743193		0.904877929	
SP011	AME	0.566997258		0.669915741		0.628002681	
SP188	ASIA	0.074118789		0.051300419		0.400486666	
SP037	EUR	0.00248503	**	0.506463813		0.287096824	

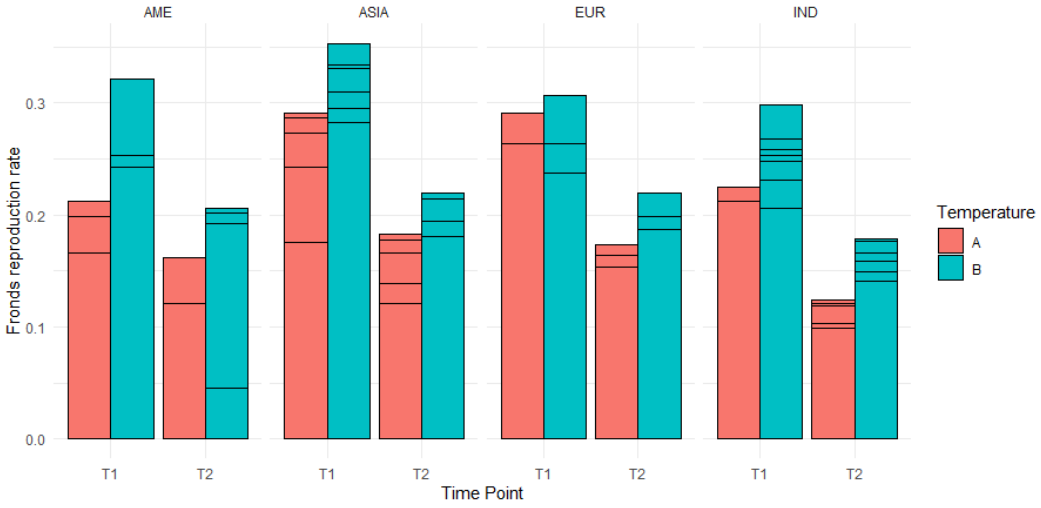

Fig S1. Fronds reproduction rates across four populations (America, Asia, Europe and India) after 6 (T1) and 12 (T2) days of bacterial inoculation. Two different temperatures were used Cologne (A, 23°C day, 13°C night) and San Marino (B, 27°C day, 13°C night).

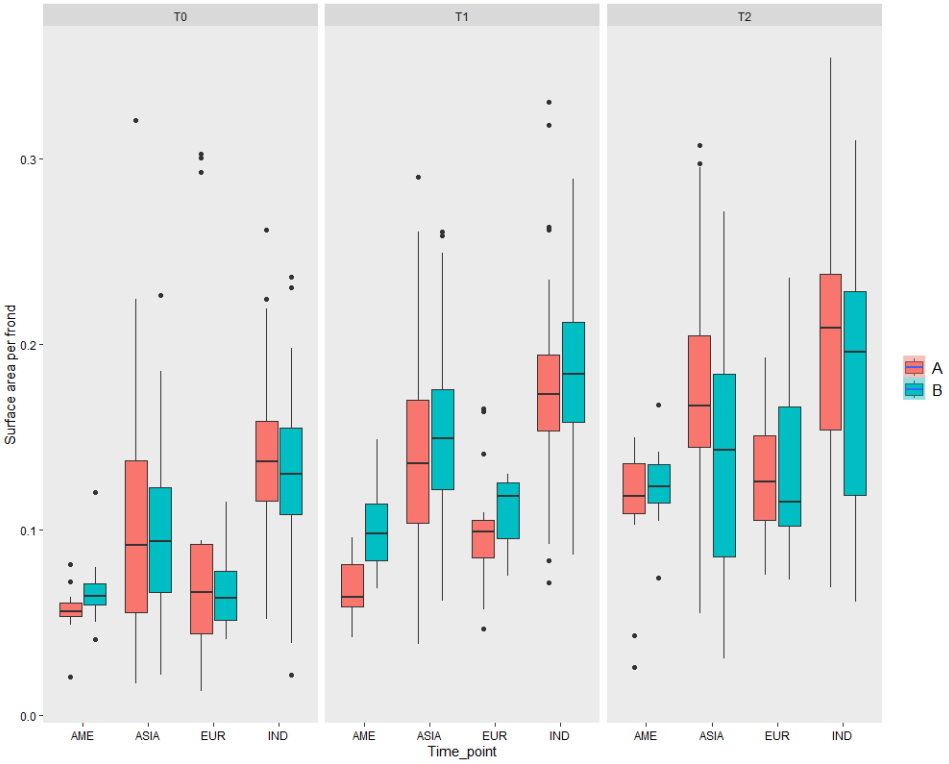

Fig S2. Fronds surface area across four populations (America, Asia, Europe and India) on the day of inoculation and after 6 (T1) and 12 (T2) days of bacterial inoculation. Two different temperatures were used Cologne (A, 23°C day, 13°C night) and San Marino (B, 27°C day, 13°C night).

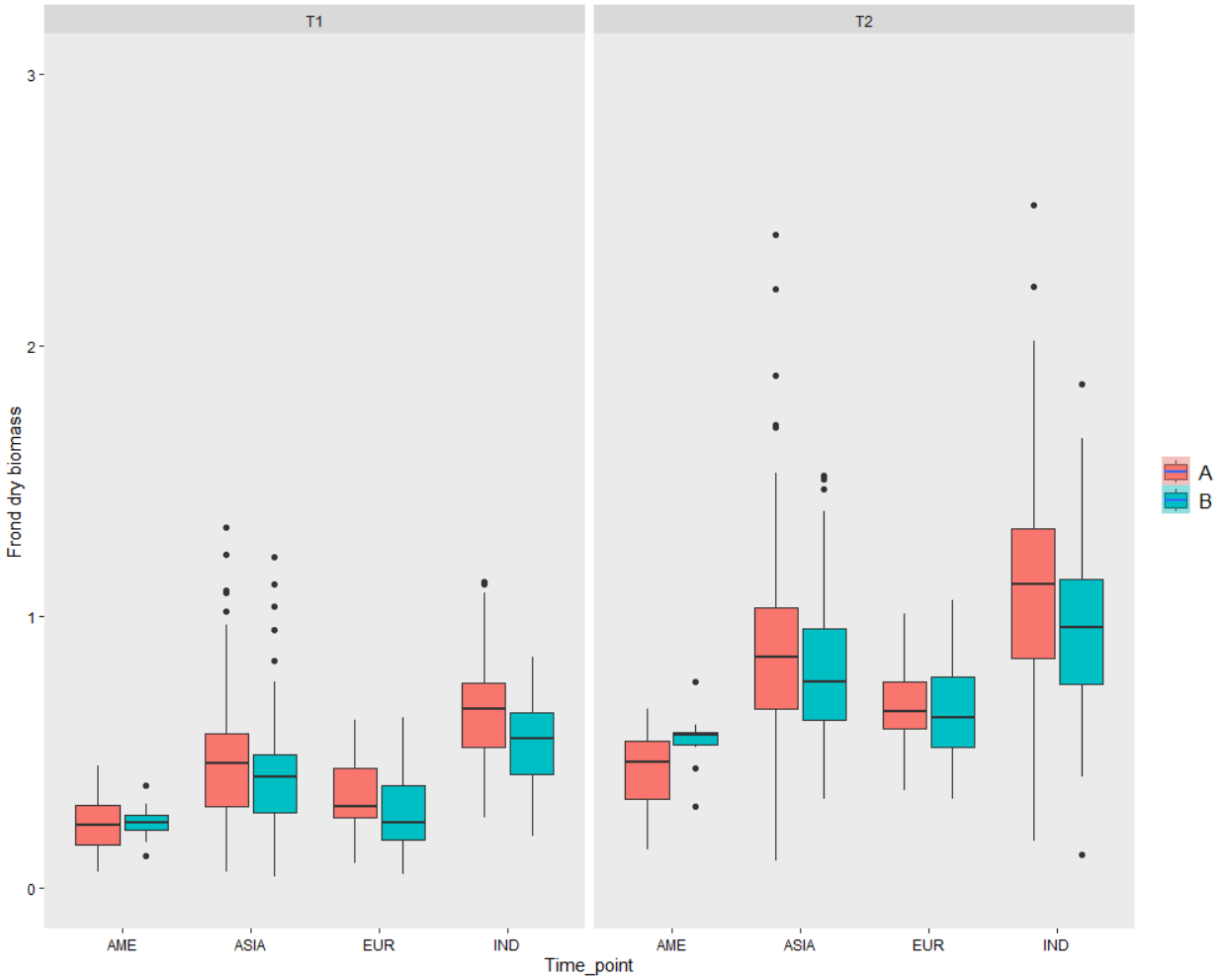

Fig S3. Fronds dry biomass across four populations (America, Asia, Europe and India) after 6 (T1) and 12 (T2) days of bacterial inoculation. Two different temperatures were used Cologne (A, 23°C day, 13°C night) and San Marino (B, 27°C day, 13°C night).