Understanding soybean root microbiome assembly along its development from both plant and soil perspectives

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Abstract

Plants have evolved intimate partnerships with microorganisms to compensate for their sessile nature and to respond to changing biotic and abiotic environmental conditions. The plant-associated microbiome greatly expands plant functions and plasticity via improved nutrient availability/uptake, enhancing plant abiotic stress tolerance, and promoting plant defense. Understanding plant microbiome assembly is crucial for leveraging their versatile benefits to the plant host and advancing the next green movement in sustainable agriculture. In this study, we comprehensively characterized soybean root-associated microbiome assembly via the 16S rRNA gene and ITS2 (internal transcribed spacer) region amplicon sequencing. Using this technology, we examined different factors influencing the soybean rhizosphere and endosphere microbiome development. Our first study examined the impacts of both plant genotype and soil type, which revealed a predominant impact of soil background in determining soybean rhizosphere microbiome assembly, while the soybean genotype plays a minor but significant role. For the next study, we investigated the role of root exudates in structuring the microbiome, specifically focusing on strigolactones. Strigolactones are a recently discovered carotenoid-derived plant hormones that play significant roles in plant-plant and plant-microbe interactions via both *in planta* and *ex planta* activities. In this study, we built three overexpression constructs targeting one biosynthesis gene and two signaling genes involving the strigolactone signaling pathway. The overexpression of these genes in soybean roots significantly impacted rhizosphere bacterial community composition. Our final study characterized soybean root microbiome assembly at three different early development stages and evaluated the relative contribution of soil-derived and seed-carried microbiome for soybean root microbiome assembly. The results indicated that soil indigenous microbes played a more determinant role for root microbiome composition in comparison to seed microbiome. During this study, we also evaluated the impact of fungicide seed treatment for this assembly process, which turned out to be insignificant for the bacterial community but significant for the fungal community. Our findings provided a comprehensive understanding of the soybean root microbiome from a systemic perspective, incorporating plant, soil and seed aspects. It will help to pave the way for microbe-assisted sustainable agriculture by optimizing and maximizing the beneficial plant-microbe interactions.

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Chapter 1 Introduction

1.1 Plant holobiont

Plants have evolved with intimate interactions with associated microorganisms, which may have facilitated the plant terrestrialization process that occurred 450 million years ago (Pirozynski and Malloch 1975). These organisms together provide mutual benefits and functions as an ecological entity referred to as a “holobiont” (Hassani, Durán, and Hacquard 2018; Vandenkoornhuyse et al. 2015; Margulis and Fester 1991). With this systemic view, plant phenotypes are being examined as a result of the multipartite interaction between plants, their associated organisms, and the environment (Müller et al. 2016). The diverse plant microbial community, collectively referred to as the plant’s second genome, is involved in diverse plant functions ranging from nutrient uptake to stress tolerance and disease resistance (Berg et al. 2014b; Berendsen, Pieterse, and Bakker 2012). Further, plants can actively structure their microbiota to dynamically adjust to the changing environment (Vandenkoornhuyse et al. 2015). Understanding the structure and function of plant microbiome assembly in response to changing biotic and abiotic environmental conditions is crucial to leverage its versatile benefits for sustainable agriculture.

1.1.1 The study of plant-microbe interactions

The plant-microbe partnership is known to be ancient, with evidence of the initial colonization of terrestrial environments by plant relying on the assistance of ancestral arbuscular mycorrhizal (Beerling 2017; Humphreys et al. 2010). This mutualistic interaction between plants and arbuscular mycorrhizal fungi (AMF) has accompanied the evolution and diversification of plant phototrophs (Vandenkoornhuyse et al. 2015; Selosse and Le Tacon 1998). Study of the symbiotic interaction between thalloid liverwort, one of the most ancient clades of land plants, and ancestral AMF revealed remarkably improved plant fitness as a consequence of the enhanced acquisition of phosphorus and nitrogen through expanded AMF colonization, with 100m to 400m mycelia for each plant (Humphreys et al. 2010). The nitrogen-fixing rhizobia are another group of bacteria that greatly facilitate plant nitrogen acquisition by forming symbiotic interactions with legumes (Philippot et al. 2013). Surprisingly, the mycorrhizal fungi and nitrogen-fixing rhizobia contributed to 80% and 75% of all nitrogen and phosphorus that respectively acquired by plants annually (Van Der Heijden, Bardgett, and Van Straalen 2008). In addition to symbiotic AMF and rhizobia, plant bacterial and fungal pathogens are another widely investigated area of plant-microbe interactions. This research has been spurred by the need to help guide plant disease management and to prevent agricultural and horticultural losses (Müller et al. 2016).

The study of plant-microbe interactions has a long history, tracing back to 1904 when the rhizosphere was first defined by Lorenz Hiltner (Hiltner 1904; Hartmann, Rothballer, and Schmid 2008). Early studies of plant-microbe interactions were largely restricted to culturable microbes (Rastogi, Coaker, and Leveau 2013). As more than 99% of the microorganisms are not culturable using standard culturing techniques, cultivation-based study prevented a comprehensive understanding of plant microbial communities (Hugenholtz 2002). Advances in next-generation sequencing technology in the early 2000s and the striking reduction in sequencing cost along with corresponding advances in computing algorithms have vastly expanded research avenues in the plant microbiome research field (Pieterse, de Jonge, and Berendsen 2016; Philippot et al. 2013; Bulgarelli et al. 2013).

1.1.2 Techniques in plant microbiome study

New multidisciplinary approaches have advanced our understanding of the importance of plant-associated microbiome (Sánchez-Cañizares et al. 2017). With the advances of next-generation sequencing, culture-independent sampling enabled in-depth analysis of plant microbial community composition and structure. Comprehensive characterization of plant microbial community composition and structure under different environmental conditions across diverse plant species has revealed some consistently conserved phylogenetic and functional aspects of plant-associated microbiomes (Shi et al. 2015; Levy, Conway, et al. 2018). Meanwhile, the rapid accumulation of microbe cultures for functional assays has provided a new annotation of microbial activities at the organism and genome levels. These greatly expanded resources for functional comparison opened new understandings of the functional specificity between plant-associated and free-living microbes (Levy, Conway, et al. 2018; Levy, Salas Gonzalez, et al. 2018). Large culture collections have also been used to design synthetic community (SynCom) experiments, providing a tractable, reduced experimental system to build a systemic understanding of plant-microbe interactions (Müller et al. 2016; Finkel et al. 2017). Plant experiments utilizing natural microbial pools, synthetic communities and individual cultures now also benefit from myriad other technologies, such as multi-omics techniques, 3D root structure imaging (e.g., X-ray micro-computed tomography scanning) as well as *in situ* plant-microbe interaction imaging using the TRIS system

(Chaparro, Badri, and Vivanco 2014; Zhalnina et al. 2018; Downie et al. 2015; Massalha et al. 2017).

1.1.3 The composition, structure, and function of the root microbiome and seed microbiome

Plant microbiome studies have focused on diverse compartments of specialized microbial communities, including those inhabiting roots (root endosphere), the whole above-ground plant (phyllosphere), leaves (leaf endosphere), stems (stem endosphere), flowers (anthosphere), seeds (spermosphere) and fruits (carposphere) (Berg et al. 2014a). Further compartments of plant-microbe interfaces include the rhizosphere, the narrow region between plant roots and surrounding soil, and the rhizoplane, which refers to microbes that inhabit the surface of plant roots. The majority of research has focused on the rhizosphere compartment due to its ecological and agricultural importance by functioning as the crucial interface mediating plant water absorption and nutrient uptake and curating diverse communication between plant and surrounding organisms (Hartmann, Rothballer, and Schmid 2008; Hiltner 1904; Berg et al. 2014a). The phyllosphere, as another crucial interface between plant and air, is a well-investigated microhabitat for plant microbiome research due to its large surface area and role as the first-line defense against air-borne pathogens (Vorholt 2012; Rastogi, Coaker, and Leveau 2013; Berg et al. 2014a). In this study, we focused on soybean-microbe interactions with an emphasis on belowground habitats, including bulk soil (i.e., surrounding soil apart from roots), rhizosphere (about 0-2mm radius surrounding roots) (Zoysa, Loganathan, and Hedley 1997), and root endosphere (inside of roots) compartments (Lundberg et al. 2012). In addition, we investigated the soybean seed microbiome with the purpose to understand the relative contribution of seed-derived microbes and soil-derived microbes for soybean root endosphere assembly.

The rhizosphere is considered to be one of the most dynamic interfaces on Earth with high numbers of microorganisms (Philippot et al. 2013). A primary interface between plant and soil microbes, the rhizosphere microbiome provided the first line of defense against root infection by soil-borne pathogens (Mendes et al. 2018). The microbial community in this region is different from that in the bulk soil in both species composition and density of microbial cells. The microbial population in the rhizosphere is comparatively higher than that of bulk soil ranging from 10^8 to 10^9 bacteria per gram (Weinert et al. 2011; Berendsen, Pieterse, and Bakker 2012). However, the taxonomic diversity of the rhizosphere microbial community is comparatively low in comparison to the inhabiting surrounding soil community as a result of the selective enrichment of specific taxa within the rhizosphere (Roesch et al. 2007).

Large scale characterization of rhizosphere microbiome under the various conditions and across numerous plant species revealed some conserved adaptions of the microbes both phylogenetically and functionally. Compared with bulk soil, a subset of the soil bacteria is typically enriched in rhizospheres, including *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, while *Acidobacteria* and *Verrucomicrobia* are commonly decreased in the rhizosphere (Müller et al. 2016). However, the rhizosphere microbiome assembly can also differ between plant species or genotypes, and the degree of rhizosphere effects (a phylogenetic community composition difference between rhizosphere and bulk soil microbiome) varies between plant species (Turner et al. 2013; Müller et al. 2016). Functional characterizations of plant microbiomes is based on many different laboratory-based functional assays as well as metagenomic sequencing and genome sequencing of isolates, in which gene sequences can be used to infer function. Specialized functional adaptiveness of plant-associated microbes in comparison to that of free-living bacteria has revealed genes related to secretion system such as T3SS and T6SS, carbohydrate metabolism and transport-related genes, genes involved in aromatic compound degradation, siderophore biosynthesis genes, chemotaxis associated genes, and flagellum biosynthesis-related genes (Levy, Salas Gonzalez, et al. 2018; Levy, Conway, et al. 2018). Microbial surface structures, such as chitin and peptidoglycan, are essential for the establishment of plant-microbe interactions by function as the signal molecules (Sánchez-Cañizares et al. 2017).

The root endosphere harbors less diverse microbial communities in comparison to the rhizosphere. Comparison of root associated microbiome in rice, maize, Arabidopsis and 30 other angiosperm species revealed that plant roots assemble distinct microbial communities between rhizosphere and endosphere microbiome (Lundberg et al. 2012; Edwards et al. 2015; Fitzpatrick et al. 2018). Endosphere microbes were mainly a subset of the rhizosphere microbiome, suggesting the endosphere is formed by discriminative recruitment or exclusion of external microbes into the root (Edwards et al. 2015; Lundberg et al. 2012). Most of the operational taxonomic units (OTUs) depleted from the rice rhizosphere are also depleted in the rhizoplane and endosphere communities, which indicate the recruitment of microbes from bulk soil to the rhizosphere acting as the first step of root microbiome assembly (Bulgarelli et al. 2013; Edwards et al. 2015). For endophytic microbes, gene functions related to stress adaptations, biofilm formation, endoglucanase, and plant cell-wall degradation were summarized as important factors determining their colonization and interaction with host plants (Hardoim, van Overbeek, and van Elsas 2008).

The microbial community within and around seeds are also of crucial importance for plant fitness by functioning as an initial source of inoculum for seedling microbiome development (Torres-Cortés et al. 2018). In contrast to the deeply investigated rhizosphere and phyllosphere microbiome via multi-omics technique, our understanding of the seed microbiome has been mainly investigated by a culture-based approach and therefore remains largely unknown (Barret et al. 2015; Nelson 2018). According to diverse surveys conducted across various plant species, the assemblage of the seed microbiome was revealed to be frequently observed in other plant organs, including *Proteobacteria*, *Actinobacteria*, *Firmicutes* or *Dothideomycetes* (Torres-Cortés et al. 2018). In terms of plant microbiome assembly, seed-associated microorganisms are hypothesized to play a more important role during the early stages of plant development, affecting germination and seedling survival, but are then outcompeted by soil indigenous microbes at later stages of plant development (Sánchez-Cañizares et al. 2017; Truyens, Weyens, and Cuypers 2015). In addition to their potential impact on plant microbiota assembly, the composition of the seed microbe influences seed preservation, seed dormancy, germination rate and disease development (Chee-Sanford et al. 2006; Goggin et al. 2015; Nelson 2018).

1.2 The importance of the plant microbiome in sustainable agriculture

Under changing climate regimes, innovative and sustainable agriculture practices focused on less input and stable yield are crucial to meet the needs of an increasing human population (Jez, Lee, and Sherp 2016; Challinor et al. 2014; Hansen, Sato, and Ruedy 2012). Modern intensified agriculture mainly relies on the cultivation of high-yield cultivars in combination with the heavy application of agrochemicals, including fertilizers and pesticides (Schlaeppi and Bulgarelli 2015). These modern agricultural practices help increase yield, however, they may also impose risks for environmental and ecological safety, including eutrophication caused when excessive nitrogen and phosphate fertilizer running off into water bodies, intensified water pollution due to intensive pesticide application, soil quality degradation and the loss of crop genetic diversity (Schlaeppi and Bulgarelli 2015; Tilman et al. 2002; Jez, Lee, and Sherp 2016).

Plants have evolved with divergent strategies to alleviate biotic and abiotic environmental stresses, including reliance on their microbial partners to survive and defend themselves against microbial invaders (Turner, James, and Poole 2013). Evidence is accumulating that the plant-associated microbiome is a key factor in plant phenotypic plasticity in response to the changing environment (Goh et al. 2013). Surprisingly, the utilization of microorganisms to stimulate plant growth in agriculture can be traced back to around 300BC, with a reference to mixing different soils as a way of ‘remedying defects and adding heart to the soil’ (Vessey 2003; Bandel and Allan Bandel 1975; Finkel et al. 2017). Modern research now focuses on characterizing the various plant growth-promoting rhizobacteria (PGPR), their underlying mechanisms of action, and the interaction of PGPR with other microbes (Dutta and Podile 2010). PGPRs promote plant growth in various ways, directly via nutrient uptake, stimulation of root growth, rhizoremediation, and alleviation of plant stresses, and, indirectly via antibiosis, induction of systemic resistance, and nutrient/niches competition with pathogens (Lugtenberg and Kamilova 2009; Bakker et al. 2018, Carvalhais, Schenk, and Dennis 2017; Bulgarelli et al. 2013). Findings in the plant microbiome field can stimulate corresponding advances in sustainable agriculture, including the development of microbial inoculants as biofertilizers, biocontrol, or stress alleviation products (Berg et al. 2014a). Crop plant breeding has mainly focused on the genetic variabilities of the plant itself for the development of more productive, disease tolerant and stress-resistant varieties, however, plant microbiome functions are usually ignored despite their indispensable importance for the next green movement (Jez, Lee, and Sherp 2016; Gopal and Gupta 2016). Methods to translate plant microbiome findings to maximize their benefits to the host plant is a crucial next step forward for sustainable agriculture.

1.3 Understand the root microbiome assembly process

Despite the tremendous benefits leveraging plant microbiomes could provide, we still have a long journey to go in terms of translating plant microbiome findings into sustainable agriculture. From plant engineering perspectives, we first need to discover crucial plant genes mediating the selective recruitment of beneficial microbes. From microbe inoculant application perspectives, we need to understand the compatibility between inoculated microbes and host plant activities, the colonization efficiency of inoculated microbes and their persistence in the context of the indigenous microbial community and local soil physio-chemical conditions (Finkel et al. 2017; Dutta and Podile 2010). To further both of those, a comprehensive understanding of plant microbiome assembly under the systemic perspective of plant-soil-microbe system will open up avenues for engineering plant microbiota for sustainable agriculture (Sánchez-Cañizares et al. 2017; Huang et al. 2019).

1.3.1 Factors shaping root microbial community

Soil type has been widely characterized as the predominant driving factor for root microbiome assembly, which is true for both the rhizosphere and endosphere microbiome (Philippot et al. 2013; Berg and Smalla 2009). For the rhizosphere microbiome assembly, most microbiota members are acquired from the surrounding soil environment functioning as the start microbe pool for the plant to recruit from (Sánchez-Cañizares et al. 2017; Bulgarelli et al. 2013). Soil physio-chemical properties (including soil nutrients, moisture, and texture) also impact the rhizosphere microbiome assembly indirectly via their impact on plant activities (Philippot et al. 2013).

Plant species differ in how they mediate rhizosphere microbiome assembly. However, the influence of plant species is comparatively small in comparison to the impact of soil type (Pieterse, de Jonge, and Berendsen 2016). Recently, not just the species but the specific plant genotype or accession has been suggested to alter microbial community composition in the rhizosphere of Arabidopsis (Micallef et al. 2009; Lundberg et al. 2012), maize (Peiffer et al. 2013), rice (Edwards et al. 2015), and soybean (Liu et al. 2019). Generally, the influence of plant genotype on rhizosphere microbiota composition is detectable but relatively weak and varies depending on soil type and plant development stage (Bulgarelli et al. 2012; Peiffer et al. 2013; İnceoğlu et al. 2011). Peiffer et al. (2013) showed a small influence of maize genotype on total variation in rhizosphere bacterial β-diversity and OTU richness across fields, but a significant fraction of maize genotype effect within each field environment (Peiffer et al. 2013). Similarly, the rhizosphere bacterial community structure showed cultivar-dependence in potato at the seedling stage, but differences disappeared in later developmental (İnceoğlu et al. 2011).

The mechanisms that plants utilize to regulate rhizosphere microbial composition and activity are not yet well understood. Important factors include photosynthate allocation strategy, root morphology, rhizodeposits amount and composition, and specific symbiotic interaction of AMF or nitrogen-fixing rhizobia (Philippot et al. 2013; Bulgarelli et al. 2013). The regulating role of root exudates has been extensively highlighted in the rhizosphere microbe assembly (Bais et al. 2006; Sasse, Martinoia, and Northen 2018; Zhalnina et al. 2018; Shi et al. 2015). By the active release of root exudates, plants selectively stimulate or inhibit particular soil microorganisms in the rhizosphere (Hartmann et al. 2009; Zhalnina et al. 2018). The amount and composition of root exudates vary considerably along developmental stages and plant age (Chaparro, Badri, and Vivanco 2014). Consequently, the development stage of the plant is indicated to be another significant factor contributing to rhizosphere microbiota variation, which has been documented in several species including potato, soybean and Arabidopsis (Micallef, Shiaris, and Colón-Carmona 2009; Xu et al. 2009; İnceoğlu et al. 2011; Chaparro, Badri, and Vivanco 2014).

1.3.2 The underlying tuning forces by the host during plant microbiome assembly

Cross-disciplinary study of the plant-soil-microbe interaction system together with advances in multi-omics technique and improvements in *in situ* image has dramatically pushed forward our understanding of plant microbiome assembly (Bakker et al. 2018). The dynamics of root exudates has been consistently revealed as one of the main manipulating factors in structuring the discriminant assembly of root associated microbiomes (Zhalnina et al. 2018; Chaparro, Badri, and Vivanco 2014). This is attributed to the divergent metabolites preference by individual microbes toward specific substrates (Zhalnina et al. 2018). Plant root exudates are comprised of many individual compounds and scientists are now teasing apart the roles of these individual substances in structuring the microbiome. For example, alteration of a triterpene biosynthesis-related gene in Arabidopsis resulted in a shifted root microbiota compared with the wild type (Huang et al. 2019). Isoflavonoids, identified as an essential modulator for soybean-rhizobium symbiosis, have also been implicated in structuring the rhizosphere bacterial community in soybean (White et al. 2015). Phenolic compounds in the root exudates were demonstrated to be essential signals for microbe assembly in the rhizosphere of Arabidopsis (Badri et al. 2013). Despite this promising progress, many root exudates have yet to be examined in terms of microbiome research.

Specific pathways involved in important secondary metabolites synthesis, nutrient starvation and plant immune system responses have illustrated as the fine-tuning factors of plant microbiomes (Stringlis et al. 2018; Huang et al. 2019; Castrillo et al.

2017; Lebeis et al. 2015). The coordinate regulations between host immunity response and nutrient starvation were demonstrated to be key players in the selection, proliferation, and interactions of microbes in the rhizosphere. For example, MYB72 transcription factor-dependent root exudation of the antimicrobial coumarin scopoletin inhibited soil-borne fungal pathogens while beneficial bacteria (involved in induced systemic resistance and iron-uptake responses) were less repressed (Stringlis et al. 2018). Similarly, the transcriptional regulator PHR1 directly stimulated beneficial microbe assisted response under phosphate starvation conditions while repressing plant immune response toward the colonization by beneficial microbes, indicating a priority of nutrient uptake over plant defense (Castrillo et al. 2017). Lebeis et al. revealed that plant defense-related hormone salicylic acid mediated the colonization by specific bacterial families into the root interior (Lebeis et al. 2015). In addition, plant pathogen resistance toward specific pathogens was revealed to play a crucial role in the recruitment/enrichment of beneficial microbes capable of antibiotic biosynthesis (Mendes et al. 2018).

1.3.3 Seed microbiome

In terms of plant microbiome assembly, there are two main pathways: horizontal transmission and vertical transmission (Torres-Cortés et al. 2018). Horizontal transmission mediates microbiota recruitment from the surrounding environment refers to microbe recruitment from the surrounding environment, i.e., from surrounding sol and aerosols/rainfall fro the rhizosphere and endosphere, respectively (Bulgarelli et al. 2013; Vacher et al. 2016; Lymperopoulou, Adams, and Lindow 2016). In contrast, vertical transmission emphasizes the acquisition and passing down of microbes from generation to generation, which has been less frequently investigated (Shade, Jacques, and Barret 2017). The seed microbiome, as the main source for microbial vertical transmission in plants, plays a crucial role in plant microbiome development during germination and emergence stage,s and has a long term impact during plant later development (Nelson 2018; Truyens, Weyens, and Cuypers 2015).

1.3.4 Microbe-microbe interactions

The functional capacity of the plant microbiome includes the complex interaction between microbes (van der Heijden and Hartmann 2016). The interactions include competition for resources, antagonistic repression, mutualism, and exchange of genetic material, all of which are known to influence microbiome composition and host health (Layeghifard, Hwang, and Guttman 2017; Hacquard et al. 2015; Stecher et al. 2012). Some taxa can be considered as “keystone”, in that they drive for microbial structure and function irrespective of their abundance (Banerjee, Schlaeppi, and van der Heijden 2018). For example, the metabolic activity of one microbe could supply the necessary substrate for the secondary colonizer, and thus facilitate the growth of other microbes (Roberts and Lindow 2014). Of Particular interest for agriculture application is the antagonistic interaction between biocontrol microbes and plant pathogen microbes.

Co-occurrence network models of microbiome provide a new perspective for understanding the dynamic assembly of plant-associated microbes, and help to guide hypothesis generations for specific microbe-microbe interactions (Poudel et al. 2016; Weiss et al. 2016). The networks also highlight hub taxa, the small number of strongly interconnected taxa. Hub taxa have disproportional importance in shaping the Arabidopsis phyllosphere microbiome based on the observation that abiotic factors act directly on ‘hub’ species, which then cascade/magnify the impact on the whole community (Agler et al. 2016).

1.4 Challenges and perspectives for plant microbiome study

Our understanding of plant microbiome assembly and function has rapidly expanded during the last decade, benefiting both from the intensive and broad ecological characterization and mechanistic understanding derived from reductionistic systems (Finkel et al. 2017). Systems-wide multi-omics technique together with advanced computational algorithms provide increasing quantities of data and have accelerated our understanding of the main driving factors involved in plant microbiome assembly (Müller et al. 2016). Isolate collections with associated function characterization as well as plant mutant resources has led to understanding of the underlying mechanism driving the dynamic plant-microbe interactions in response to the changing environment (Finkel et al. 2017). Considering the dynamic manipulation by the plant on the associated microbiome in response to changing nutrient and surrounding pathogen pressure, the exploration of plant-microbe interactions needs to simultaneously take into account of the composition of introduced microbial consortia and their compatibility with plant genotypes and soil types (Bakker et al. 2018). In addition, functional understanding of plant microbiome from both the individual microbe and community level perspectives is of great importance, with an emphasis on the underlying microbe-microbe interactions in structuring robust and stable beneficial plant-microbe interactions.

One of our ultimate goals for plant microbiome study is to exploit the versatile benefits supplied by PGPRs to maximize crop performance in the field (Finkel et al.

2017). Mostly, the PGPR effects of individual microbiota were tested in the lab under gnotobiotic conditions (Forni, Duca, and Glick 2017), and the efficiency of their beneficial impacts tend to be diminished when applied into field conditions (Finkel et al. 2017). The ultimate benefits that PGPRs could deliver to crop plants depends on a series of factors, including their invasion and persistence capacity when inoculated in native soil, their compatibility with the host plant as well as the interactions with indigenous microbes (Vejan et al. 2016; Dutta and Podile 2010). The effectiveness of inoculating individual PGPR can be limited due to its limited capacity to maintain high abundance in natural soil, while bacteria consortia with higher richness usually achieve better outcomes due to synergistic effects between inocula(Finkel et al. 2017). Consequently, a systemic understanding of the dynamic interaction between indigenous microbes and the inoculant's consortia is an important next step to further plant microbiome study. With the increasing culture collections, SynCom based ecological understanding of plant-microbe interactions and microbe-microbe interactions will shine more light on these questions.

In contrast to the rapid accumulation in root microbiome research, our understanding of the seed microbiome and spermosphere microbiome is less developed despite its crucial importance in plant establishment and health as well as its great potential in inoculant based agricultural application. Large scale characterization of seed microbiome composition and structure across different plant species growing under different climate and soil conditions is needed. Similarly, the question of how the seed microbiome is vertically transmitted from generation to generation, and how seed carried pathogens or beneficial microbes influence the seedling establishment and later growth are critical.

1.5 Research questions and hypotheses

The soybean is one of the most important crops worldwide by serving as a vital source of protein and vegetable oil. Understanding the overall root microbial community structure and dynamics is crucial for maximizing the beneficial soybean-microbe interactions considering the universal co-existence and the diverse interactions between rhizobia and other microbes. Our overall goal is to profile the taxonomic diversity of the soybean root microbiome from an integrative perspective encompassing soybean genotypes, soybean signaling pathways, and soil types at various soybean development stages. Our three objectives emphasize these different specific aspects of soybean root microbiome assembly (Fig. 1.1):

Objective #1: How does the soybean genotype and soil microbial pool impact microbial community assembly in the rhizosphere? (Chapter 2)

(i) Hypothesis 1a: The indigenous microbes in the soil impacts the rhizosphere microbiome assembly.

(ii) Hypothesis 1b: The soybean genotype cooperatively impacts the rhizosphere microbiome assembly.

(iii) Hypothesis 1c: The indigenous microbes in the soil and the soybean genotype interact to influence the rhizosphere microbiome assembly, with soil microbes as the primary driving factor in comparison to soybean genotypes.

Objective 2: What role do strigolactones play in the soybean rhizosphere microbiome assembly? (Chapter 3)

(i) Hypothesis 2a: Overexpression of the strigolactone biosynthesis gene Max1 influences the taxonomic composition of the soybean rhizosphere microbiome.

(ii) Hypothesis 2b: Overexpression of the strigolactone perception gene Max2 influences the taxonomic composition of the soybean rhizosphere microbiome.

(iii) Hypothesis 2c: Overexpression of the strigolactone perception gene D14 influences the taxonomic composition of the soybean rhizosphere microbiome.

Objective 3: Who is driving soybean root microbiome assembly and development - microbes from the seed, the soil indigenous community, or both? Does seed fungicide application disrupt this process? (Chapter 4)

(i) Hypothesis 3a: The soybean rhizosphere microbiome will be assembled from the surrounding soil microbial pool and not the seed carried microbes.

(ii) Hypothesis 3b: The soybean root endosphere microbiome will be assembled simultaneously from the surrounding soil and seed carried microbes.

(iii) Hypothesis 3c: The relative contribution of seed-borne microbes will gradually decrease over time during soybean development.

(iv) Hypothesis 3d: Fungicide application to seeds prior to sowing will disrupt bacterial and fungal communities in the root rhizosphere.

(v) Hypothesis 3e: Fungicide application to seeds prior to sowing will disrupt bacterial and fungal communities in the root endosphere.

1.6 Approaches

For the microcosm experiments, soybeans were grown in the greenhouse under controlled conditions in pots filled with field soil. At the flowering stages, soybean rhizosphere soil samples were gently collected together with bulk soil (from pots without soybean plants). Soil DNA was extracted using the Powersoil extraction kit following the protocol. To profile the bacteria and fungi communities, sequencing libraries targeting the 16S rRNA gene V3-V4 and ITS2 (internal transcribed spacer) region were prepared based on a two-step PCR process and Illumina Miseq 16S metagenomic sequencing library preparation pipelines. The prepared libraries were sequenced on an Illumina MiSeq for 300 base paired-end sequencing.

Mothur software was used to pre-process the sequencing data, including contigs assembly, quality control, chimera detection, and discard, alignment, OTU (operational taxonomic unit) clustering, and taxonomy classification. The Mothur generated feature table associated with corresponding taxonomy information were used as input for community analysis in R. Community analysis included diversity calculations, dimensional reduction, variance analysis, and visualization using R packages, including Phyloseq, vegan, ggplot2, pheatmap, reshape2, dplyr, etc. Differential abundance analysis was conducted using LefSe software, while, Tax4Fun/Tax4Fun2 and PICRUST2 were used to predict bacterial meta-function based on taxonomy information. FUNGuilds software was used to predict the fungal trophic groups based on corresponding taxonomy information. In terms of data arrangement and file formatting, both shell and R commands were used.

1.7 Rationale and importance

A comprehensive understanding of the root microbiome from both plant and soil aspects is of crucial importance for maximizing the beneficial plant-microbe interaction while eliminating the deleterious plant-microbe interactions. Our understanding in this field is accumulating very rapidly but has largely focused on model species instead of production crops. Mechanistic findings from the model plants, e.g, Arabidopsis, provides a broad understanding of root microbiome assembly. However, plants are featured with species-specific manipulation on associated microbe depending on their evolutionary and ecological differences. Soybean, as an important legume crop, has been well studied in terms of its symbiotic interaction with nitrogen-fixing rhizobia. However, rhizobia coexist with a complex microbial community co-exist with rhizobia in the soybean rhizosphere, endosphere, and even within nodules. These co-inhabitants of microbes not only dynamically impact the relative abundance of rhizobia but also are biologically important by functioning as the synergistic partners of rhizobia to enhance nodulation and by promoting legume survival especially under stressed environmental conditions (Martínez-Hidalgo and Hirsch 2017). It has been revealed that cultivated soybeans were domesticated from their wild annual progenitor (*Glycine soja* Sieb. & Zucc.) about five thousand years ago in China (Carter et al. 2004). During the soybean domestication process, 81% of the rare alleles, which may constitute genetic variation essential for biotic/abiotic resistance, from Glycine soja were lost in the modern elite cultivars (Hyten et al. 2006; Zhou et al. 2015). Whether the loss of the aforementioned genetic diversity in modern soybean cultivars deteriorated the capacity for recruiting beneficial microbes, we still lack information.

Strigolactones as an important class of hormones playing multi-functions both *in planta* and *ex planta*, including plant morphology to plant-plant and plant-microbe communications, and plant abiotic tolerance. Strigolactones are actively exuded from plant roots and were recently found to induce AMF formation and potentially legume nodulation. As signaling molecules, they may mediate more broad plant-microbe communication in the rhizosphere and act as the first step of microbe selection, which may contribute to the enhanced AMF formation or nodulation. An integrative understanding of strigolactone impacts on soybean root microbiome could help to dissect their direct and indirect impact on plant performance in association with rhizobia and AMF, which is important in guiding advanced genetic engineering.

A comprehensive understanding of soybean microbiome assembly must also take into account early development and the relative contribution of both microbial horizontal transmission from the soil and vertical transmission from seed. Early development is important for maximizing the potential benefits of nitrogen-fixing rhizobia and other PGPRs. Fungicide seed treatment is a common modern agricultural practice to eliminate yield loss caused by pathogens. It is currently not clear if this practice directly or indirectly disturbs the beneficial soybean-microbe interaction, and investigations are rarely conducted in field conditions.

Reference

Agler, Matthew T., Jonas Ruhe, Samuel Kroll, Constanze Morhenn, Sang-Tae Kim, Detlef Weigel, and Eric M. Kemen. 2016. “Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation.” PLoS Biology 14 (1): e1002352.

Badri, Dayakar V., Jacqueline M. Chaparro, Ruifu Zhang, Qirong Shen, and Jorge M. Vivanco. 2013. “Application of Natural Blends of Phytochemicals Derived from the Root Exudates of Arabidopsis to the Soil Reveal That Phenolic-Related Compounds Predominantly Modulate the Soil Microbiome.” The Journal of Biological Chemistry 288 (7): 4502–12.

Bais, Harsh P., Tiffany L. Weir, Laura G. Perry, Simon Gilroy, and Jorge M. Vivanco. 2006. “The Role of Root Exudates in Rhizosphere Interactions with Plants and Other Organisms.” Annual Review of Plant Biology 57: 233–266.

Bakker, Peter A. H. M., Corné M. J. Pieterse, Ronnie de Jonge, and Roeland L. Berendsen. 2018. “The Soil-Borne Legacy.” Cell 172 (6): 1178–80.

Bandel, V. Allan. 1975. “Soil Fertility and Fertilizers.” Soil Science Society of America Journal 39 (6): vi–viii.

Banerjee, Samiran, Klaus Schlaeppi, and Marcel G. A. van der Heijden. 2018. “Keystone Taxa as Drivers of Microbiome Structure and Functioning.” Nature Reviews. Microbiology 16 (9): 567–76.

Barret, Matthieu, Martial Briand, Sophie Bonneau, Anne Préveaux, Sophie Valière, Olivier Bouchez, Gilles Hunault, Philippe Simoneau, and Marie-Agnès Jacquesa. 2015. “Emergence Shapes the Structure of the Seed Microbiota.” Applied and Environmental Microbiology 81 (4): 1257–66.

Beerling, David. 2017. The Emerald Planet: How Plants Changed Earth’s History. Oxford University Press.

Berendsen, Roeland L., Corné M. J. Pieterse, and Peter A. H. M. Bakker. 2012. “The Rhizosphere Microbiome and Plant Health.” Trends in Plant Science 17 (8): 478–86.

Berg, Gabriele, Martin Grube, Michael Schloter, and Kornelia Smalla. 2014a. “Unraveling the Plant Microbiome: Looking Back and Future Perspectives.” Frontiers in Microbiology 5 (June): 148.

Berg, Gabriele, Martin Grube, Michael Schloter, and Kornelia Smalla. 2014b. “The Plant Microbiome and Its Importance for Plant and Human Health.” Frontiers in Microbiology 5 (September): 491.

Berg, Gabriele, and Kornelia Smalla. 2009. “Plant Species and Soil Type Cooperatively Shape the Structure and Function of Microbial Communities in the Rhizosphere.” FEMS Microbiology Ecology 68 (1): 1–13.

Bulgarelli, Davide, Matthias Rott, Klaus Schlaeppi, Emiel Ver Loren van Themaat, Nahal Ahmadinejad, Federica Assenza, Philipp Rauf, et al. 2012. “Revealing Structure and Assembly Cues for Arabidopsis Root-Inhabiting Bacterial Microbiota.” Nature 488

(7409): 91–95.

Bulgarelli, Davide, Klaus Schlaeppi, Stijn Spaepen, Emiel Ver Loren van Themaat, and Paul Schulze-Lefert. 2013. “Structure and Functions of the Bacterial Microbiota of Plants.” Annual Review of Plant Biology 64 (January): 807–38.

Carter, T. E., R. Nelson, C. H. Sneller, Z. Cui, H. R. Boerma, and J. E. Specht. 2004. “Soybeans: Improvement, Production, and Uses.” Edited by H. R. Boerma and J. E. Specht. Madison: American Society of Agronomy.

Carvalhais, Lilia C., Peer M. Schenk, and Paul G. Dennis. 2017. “Jasmonic Acid Signalling and the Plant Holobiont.” Current Opinion in Microbiology 37: 42–47.

Castrillo, Gabriel, Paulo José Pereira Lima Teixeira, Sur Herrera Paredes, Theresa F. Law, Laura de Lorenzo, Meghan E. Feltcher, Omri M. Finkel, et al. 2017. “Root Microbiota Drive Direct Integration of Phosphate Stress and Immunity.” Nature 543 (7646): 513–18.

Challinor, A. J., J. Watson, D. B. Lobell, S. M. Howden, D. R. Smith, and N. Chhetri. 2014. “A Meta-Analysis of Crop Yield under Climate Change and Adaptation.” Nature Climate Change 4 (March): 287.

Chaparro, Jacqueline M., Dayakar V. Badri, and Jorge M. Vivanco. 2014. “Rhizosphere Microbiome Assemblage Is Affected by Plant Development.” The ISME Journal 8 (4): 790–803.

Chee-Sanford, Joanne C., Martin M. Williams, Adam S. Davis, and Gerald K. Sims.

2006. “Do Microorganisms Influence Seed-Bank Dynamics?” Weed Science 54 (3): 575–87.

Downie, H. F., M. O. Adu, S. Schmidt, W. Otten, L. X. Dupuy, P. J. White, and T. A. Valentine. 2015. “Challenges and Opportunities for Quantifying Roots and Rhizosphere Interactions through Imaging and Image Analysis.” Plant, Cell & Environment 38 (7): 1213–32.

Dutta, Swarnalee, and Appa Rao Podile. 2010. “Plant Growth Promoting Rhizobacteria (PGPR): The Bugs to Debug the Root Zone.” Critical Reviews in Microbiology 36 (3): 232–44.

Edwards, Joseph, Cameron Johnson, Christian Santos-Medellín, Eugene Lurie, Natraj Kumar Podishetty, Srijak Bhatnagar, Jonathan A. Eisen, and Venkatesan Sundaresan. 2015. “Structure, Variation, and Assembly of the Root-Associated Microbiomes of Rice.” Proceedings of the National Academy of Sciences of the United States of America 112 (8): E911–20.

Finkel, Omri M., Gabriel Castrillo, Sur Herrera Paredes, Isai Salas González, and Jeffery L. Dangl. 2017. “Understanding and Exploiting Plant Beneficial Microbes.” Current Opinion in Plant Biology 38 (August): 155–63.

Fitzpatrick, Connor R., Julia Copeland, Pauline W. Wang, David S. Guttman, Peter M. Kotanen, and Marc T. J. Johnson. 2018. “Assembly and Ecological Function of the Root Microbiome across Angiosperm Plant Species.” Proceedings of the National Academy of Sciences of the United States of America 115 (6): E1157–65.

Forni, Cinzia, Daiana Duca, and Bernard R. Glick. 2017. “Mechanisms of Plant Response to Salt and Drought Stress and Their Alteration by Rhizobacteria.” Plant and Soil 410 (1): 335–56.

Goggin, Danica E., R. J. Neil Emery, Leonid V. Kurepin, and Stephen B. Powles. 2015. “A Potential Role for Endogenous Microflora in Dormancy Release, Cytokinin Metabolism and the Response to Fluridone in Lolium Rigidum Seeds.” Annals of Botany 115 (2): 293–301.

Goh, Chooi-Hua, Debora F. Veliz Vallejos, Adrienne B. Nicotra, and Ulrike Mathesius. 2013. “The Impact of Beneficial Plant-Associated Microbes on Plant Phenotypic Plasticity.” Journal of Chemical Ecology 39 (7): 826–39.

Gopal, Murali, and Alka Gupta. 2016. “Microbiome Selection Could Spur Next-Generation Plant Breeding Strategies.” Frontiers in Microbiology 7: 1971.

Hacquard, Stéphane, Ruben Garrido-Oter, Antonio González, Stijn Spaepen, Gail Ackermann, Sarah Lebeis, Alice C. McHardy, et al. 2015. “Microbiota and Host Nutrition across Plant and Animal Kingdoms.” Cell Host & Microbe 17(5): 603–16.

Hansen, James, Makiko Sato, and Reto Ruedy. 2012. “Perception of Climate Change.” Proceedings of the National Academy of Sciences of the United States of America 109 (37): E2415–23.

Hardoim, Pablo R., Leo S. van Overbeek, and Jan Dirk van Elsas. 2008. “Properties of Bacterial Endophytes and Their Proposed Role in Plant Growth.” Trends in

Microbiology 16 (10): 463–71.

Hartmann, Anton, Michael Rothballer, and Michael Schmid. 2008. “Lorenz Hiltner, a Pioneer in Rhizosphere Microbial Ecology and Soil Bacteriology Research.” Plant and Soil 312 (1): 7–14.

Hartmann, Anton, Michael Schmid, Diederik van Tuinen, and Gabriele Berg. 2009. “Plant-Driven Selection of Microbes.” Plant and Soil 321 (1): 235–57.

Hassani, M. Amine, Paloma Durán, and Stéphane Hacquard. 2018. “Microbial Interactions within the Plant Holobiont.” Microbiome 6 (1): 58.

Heijden, Marcel G. A. van der, and Martin Hartmann. 2016. “Networking in the Plant Microbiome.” PLoS Biology 14 (2): E1002378.

Hiltner, Lorenz 1904. “Uber Nevere Erfahrungen Und Probleme Auf Dem Gebiet Der Boden Bakteriologie Und Unter Besonderer Beurchsichtigung Der Grundungung Und Broche.” Arbeit. Deut. Landw. Ges. Berlin 98: 59–78.

Huang, Ancheng C., Ting Jiang, Yong-Xin Liu, Yue-Chen Bai, James Reed, Baoyuan Qu, Alain Goossens, Hans-Wilhelm Nützmann, Yang Bai, and Anne Osbourn. 2019. “A Specialized Metabolic Network Selectively Modulates Arabidopsis Root Microbiota.” Science 364 (6440): eaau6389.

Hugenholtz, Philip. 2002. “Exploring Prokaryotic Diversity in the Genomic Era.” Genome Biology 3 (2): Reviews0003.

Humphreys, Claire P., Peter J. Franks, Mark Rees, Martin I. Bidartondo, Jonathan R.

Leake, and David J. Beerling. 2010. “Mutualistic Mycorrhiza-like Symbiosis in the Most Ancient Group of Land Plants.” Nature Communications 1 (November): 103.

Hyten, David L., Qijian Song, Youlin Zhu, Ik-Young Choi, Randall L. Nelson, Jose M. Costa, James E. Specht, Randy C. Shoemaker, and Perry B. Cregan. 2006. “Impacts of Genetic Bottlenecks on Soybean Genome Diversity.” Proceedings of the National Academy of Sciences of the United States of America 103 (45): 16666–71.

İnceoğlu, Özgül, Waleed Abu Al-Soud, Joana Falcão Salles, Alexander V. Semenov, and Jan Dirk van Elsas. 2011. “Comparative Analysis of Bacterial Communities in a Potato Field as Determined by Pyrosequencing.” PloS One 6 (8): e23321.

Jez, Joseph M., Soon Goo Lee, and Ashley M. Sherp. 2016. “The next Green Movement: Plant Biology for the Environment and Sustainability.” Science 353 (6305): 1241–44.

Layeghifard, Mehdi, David M. Hwang, and David S. Guttman. 2017. “Disentangling Interactions in the Microbiome: A Network Perspective.” Trends in Microbiology 25 (3): 217–28.

Lebeis, Sarah L., Sur Herrera Paredes, Derek S. Lundberg, Natalie Breakfield, Jase Gehring, Meredith McDonald, Stephanie Malfatti, et al. 2015. “Salicylic Acid Modulates Colonization of the Root Microbiome by Specific Bacterial Taxa.” Science 349 (6250): 860–64.

Levy, Asaf, Jonathan M. Conway, Jeffery L. Dangl, and Tanja Woyke. 2018.

“Elucidating Bacterial Gene Functions in the Plant Microbiome.” Cell Host & Microbe 24 (4): 475–85.

Levy, Asaf, Isai Salas Gonzalez, Maximilian Mittelviefhaus, Scott Clingenpeel, Sur Herrera Paredes, Jiamin Miao, Kunru Wang, et al. 2018. “Genomic Features of Bacterial Adaptation to Plants.” Nature Genetics 50 (1): 138–50.

Liu, Fang, Tarek Hewezi, Sarah L. Lebeis, Vince Pantalone, Parwinder S. Grewal, and Margaret E. Staton. 2019. “Soil Indigenous Microbiome and Plant Genotypes Cooperatively Modify Soybean Rhizosphere Microbiome Assembly.” BMC Microbiology 19 (1): 201.

Lugtenberg, Ben, and Faina Kamilova. 2009. “Plant-Growth-Promoting Rhizobacteria.” Annual Review of Microbiology 63: 541–56.

Lundberg, Derek S., Sarah L. Lebeis, Sur Herrera Paredes, Scott Yourstone, Jase Gehring, Stephanie Malfatti, Julien Tremblay, et al. 2012. “Defining the Core Arabidopsis Thaliana Root Microbiome.” Nature 488 (7409): 86–90.

Lymperopoulou, Despoina S., Rachel I. Adams, and Steven E. Lindow. 2016. “Contribution of Vegetation to the Microbial Composition of Nearby Outdoor Air.” Applied and Environmental Microbiology 82 (13): 3822–33.

Margulis, Lynn, and Rene Fester, eds. 1991. “Symbiosis as a source of evolutionary innovation: speciation and morphogenesis.” MIT Press.

Massalha, Hassan, Elisa Korenblum, Sergey Malitsky, Orr H. Shapiro, and Asaph

Aharoni. 2017. “Live Imaging of Root–bacteria Interactions in a Microfluidics Setup.” Proceedings of the National Academy of Sciences of the United States of America 114 (17): 4549–54.

Mendes, Lucas William, Jos M. Raaijmakers, Mattias de Hollander, Rodrigo Mendes, and Siu Mui Tsai. 2018. “Influence of Resistance Breeding in Common Bean on Rhizosphere Microbiome Composition and Function.” The ISME Journal 12 (1): 212–24.

Micallef, Shirley A., Michael P. Shiaris, and Adán Colón-Carmona. 2009. “Influence of Arabidopsis Thaliana Accessions on Rhizobacterial Communities and Natural Variation in Root Exudates.” Journal of Experimental Botany 60 (6): 1729–42.

Müller, Daniel B., Christine Vogel, Yang Bai, and Julia A. Vorholt. 2016. “The Plant Microbiota: Systems-Level Insights and Perspectives.” Annual Review of Genetics 50 (November): 211–34.

Nelson, Eric B. 2018. “The Seed Microbiome: Origins, Interactions, and Impacts.” Plant and Soil 422 (1): 7–34.

Peiffer, Jason A., Aymé Spor, Omry Koren, Zhao Jin, Susannah Green Tringe, Jeffery L. Dangl, Edward S. Buckler, and Ruth E. Ley. 2013. “Diversity and Heritability of the Maize Rhizosphere Microbiome under Field Conditions.” Proceedings of the National Academy of Sciences of the United States of America 110 (16): 6548–53.

Philippot, Laurent, Jos M. Raaijmakers, Philippe Lemanceau, and Wim H. van der

Putten. 2013. “Going back to the Roots: The Microbial Ecology of the Rhizosphere.” Nature Reviews. Microbiology 11 (11): 789–99.

Pieterse, Corné M. J., Ronnie de Jonge, and Roeland L. Berendsen. 2016. “The Soil-Borne Supremacy.” Trends in Plant Science 21 (3): 171–73.

Pirozynski, K. A., and D. W. Malloch. 1975. “The Origin of Land Plants: A Matter of Mycotrophism.” Bio Systems 6 (3): 153–64.

Poudel, R., A. Jumpponen, D. C. Schlatter, T. C. Paulitz, B. B. Mcspadden Gardener, L. L. Kinkel, and K. A. Garrett. 2016. “Microbiome Networks: A Systems Framework for Identifying Candidate Microbial Assemblages for Disease Management.” Phytopathology 106 (10): 1083–96.

Rastogi, Gurdeep, Gitta L. Coaker, and Johan H. J. Leveau. 2013. “New Insights into the Structure and Function of Phyllosphere Microbiota through High-Throughput Molecular Approaches.” FEMS Microbiology Letters 348 (1): 1–10.

Rincon-Florez, Vivian A., Lilia C. Carvalhais, and Peer M. Schenk. 2013. “Culture-Independent Molecular Tools for Soil and Rhizosphere Microbiology.” Diversity 5 (3): 581–612.

Roberts, Elizabeth, and Steven Lindow. 2014. “Loline Alkaloid Production by Fungal Endophytes of Fescue Species Select for Particular Epiphytic Bacterial Microflora.” The ISME Journal 8 (2): 359–68.

Roesch, Luiz F. W., Roberta R. Fulthorpe, Alberto Riva, George Casella, Alison K. M.

Hadwin, Angela D. Kent, Samira H. Daroub, Flavio A. O. Camargo, William G. Farmerie, and Eric W. Triplett. 2007. “Pyrosequencing Enumerates and Contrasts Soil Microbial Diversity.” The ISME Journal 1 (4): 283–90.

Sánchez-Cañizares, Carmen, Beatriz Jorrín, Philip S. Poole, and Andrzej Tkacz. 2017. “Understanding the Holobiont: The Interdependence of Plants and Their Microbiome.” Current Opinion in Microbiology 38: 188–96.

Sasse, Joelle, Enrico Martinoia, and Trent Northen. 2018. “Feed Your Friends: Do Plant Exudates Shape the Root Microbiome?” Trends in Plant Science 23 (1): 25–41.

Schlaeppi, Klaus, and Davide Bulgarelli. 2015. “The Plant Microbiome at Work.” Molecular Plant-Microbe Interactions: MPMI 28 (3): 212–17.

Selosse, M. A., and F. Le Tacon. 1998. “The Land Flora: A Phototroph-Fungus Partnership?” Trends in Ecology & Evolution 13 (1): 15–20.

Shade, Ashley, Marie-Agnès Jacques, and Matthieu Barret. 2017. “Ecological Patterns of Seed Microbiome Diversity, Transmission, and Assembly.” Current Opinion in Microbiology 37 (June): 15–22.

Shi, Shengjing, Erin Nuccio, Donald J. Herman, Ruud Rijkers, Katerina Estera, Jiabao Li, Ulisses Nunes da Rocha, et al. 2015. “Successional Trajectories of Rhizosphere Bacterial Communities over Consecutive Seasons.” mBio 6 (4): e00746.

Stecher, Bärbel, Rémy Denzler, Lisa Maier, Florian Bernet, Mandy J. Sanders, Derek J. Pickard, Manja Barthel, et al. 2012. “Gut Inflammation Can Boost Horizontal Gene

Transfer between Pathogenic and Commensal Enterobacteriaceae.” Proceedings of the National Academy of Sciences of the United States of America 109 (4): 1269–74.

Stringlis, Ioannis A., Ke Yu, Kirstin Feussner, Ronnie de Jonge, Sietske Van Bentum, Marcel C. Van Verk, Roeland L. Berendsen, Peter A. H. M. Bakker, Ivo Feussner, and Corné M. J. Pieterse. 2018. “MYB72-Dependent Coumarin Exudation Shapes Root Microbiome Assembly to Promote Plant Health.” Proceedings of the National Academy of Sciences of the United States of America 115 (22): E5213–22.

Tilman, David, Kenneth G. Cassman, Pamela A. Matson, Rosamond Naylor, and Stephen Polasky. 2002. “Agricultural Sustainability and Intensive Production Practices.” Nature 418 (6898): 671–77.

Torres-Cortés, Gloria, Sophie Bonneau, Olivier Bouchez, Clémence Genthon, Martial Briand, Marie-Agnès Jacques, and Matthieu Barret. 2018. “Functional Microbial Features Driving Community Assembly During Seed Germination and Emergence.” Frontiers in Plant Science 9: 902.

Truyens, S., N. Weyens, and A. Cuypers. 2015. “Bacterial Seed Endophytes: Genera, Vertical Transmission and Interaction with Plants.” The Environmentalist 7 (1): 40–50.

Turner, Thomas R., Euan K. James, and Philip S. Poole. 2013. “The Plant Microbiome.” Genome Biology 14 (6): 209.

Turner, Thomas R., Karunakaran Ramakrishnan, John Walshaw, Darren Heavens, Mark Alston, David Swarbreck, Anne Osbourn, Alastair Grant, and Philip S. Poole. 2013. “Comparative Metatranscriptomics Reveals Kingdom Level Changes in the Rhizosphere Microbiome of Plants.” The ISME Journal 7 (12): 2248–58.

Vacher, Corinne, Arndt Hampe, Annabel J. Porté, Ursula Sauer, Stéphane Compant, and Cindy E. Morris. 2016. “The Phyllosphere: Microbial Jungle at the Plant–Climate Interface.” Annual Review of Ecology, Evolution, and Systematics 47 (1): 1–24.

Vandenkoornhuyse, Philippe, Achim Quaiser, Marie Duhamel, Amandine Le Van, and Alexis Dufresne. 2015. “The Importance of the Microbiome of the Plant Holobiont.” The New Phytologist 206 (4): 1196–1206.

Van Der Heijden, Marcel G. A., Richard D. Bardgett, and Nico M. Van Straalen. 2008. “The Unseen Majority: Soil Microbes as Drivers of Plant Diversity and Productivity in Terrestrial Ecosystems.” Ecology Letters 11 (3): 296–310.

Vejan, Pravin, Rosazlin Abdullah, Tumirah Khadiran, Salmah Ismail, and Amru Nasrulhaq Boyce. 2016. “Role of Plant Growth Promoting Rhizobacteria in Agricultural Sustainability - A Review.” Molecules 21 (5): 573.

Vessey, J. Kevin. 2003. “Plant Growth Promoting Rhizobacteria as Biofertilizers.” Plant and Soil 255 (2): 571–86.

Vorholt, Julia A. 2012. “Microbial Life in the Phyllosphere.” Nature Reviews.

Microbiology 10 (12): 828–40.

Weinert, Nicole, Yvette Piceno, Guo-Chun Ding, Remo Meincke, Holger Heuer, Gabriele Berg, Michael Schloter, Gary Andersen, and Kornelia Smalla. 2011. “PhyloChip Hybridization Uncovered an Enormous Bacterial Diversity in the Rhizosphere of Different Potato Cultivars: Many Common and Few Cultivar-Dependent Taxa.” FEMS Microbiology Ecology 75 (3): 497–506.

Weiss, Sophie, Will Van Treuren, Catherine Lozupone, Karoline Faust, Jonathan Friedman, Ye Deng, Li Charlie Xia, et al. 2016. “Correlation Detection Strategies in Microbial Data Sets Vary Widely in Sensitivity and Precision.” The ISME Journal 10 (7): 1669–81.

White, Laura J., Karuppaiyan Jothibasu, R. Neil Reese, Volker S. Brözel, and Senthil Subramanian. 2015. “Spatio Temporal Influence of Isoflavonoids on Bacterial Diversity in the Soybean Rhizosphere.” Molecular Plant-Microbe Interactions: MPMI 28 (1): 22–29.

Xu, Yanxia, Guanghua Wang, Jian Jin, Junjie Liu, Qiuying Zhang, and Xiaobing Liu. 2009. “Bacterial Communities in Soybean Rhizosphere in Response to Soil Type, Soybean Genotype, and Their Growth Stage.” Soil Biology and Biochemistry 41(5): 919–25.

Zhalnina, Kateryna, Katherine B. Louie, Zhao Hao, Nasim Mansoori, Ulisses Nunes da Rocha, Shengjing Shi, Heejung Cho, et al. 2018. “Dynamic Root Exudate Chemistry and Microbial Substrate Preferences Drive Patterns in Rhizosphere Microbial

Community Assembly.” Nature Microbiology 3 (4): 470–80.

Zhou, Zhengkui, Yu Jiang, Zheng Wang, Zhiheng Gou, Jun Lyu, Weiyu Li, Yanjun Yu, et al. 2015. “Resequencing 302 Wild and Cultivated Accessions Identifies Genes Related to Domestication and Improvement in Soybean.” Nature Biotechnology 33 (4): 408–14.

Zoysa, A. K. N., P. Loganathan, and M. J. Hedley. 1997. “A Technique for Studying Rhizosphere Processes in Tree Crops: Soil Phosphorus Depletion around Camellia (Camellia Japonica L.) Roots.” Plant and Soil 190 (2): 253–65.

Appendices 1

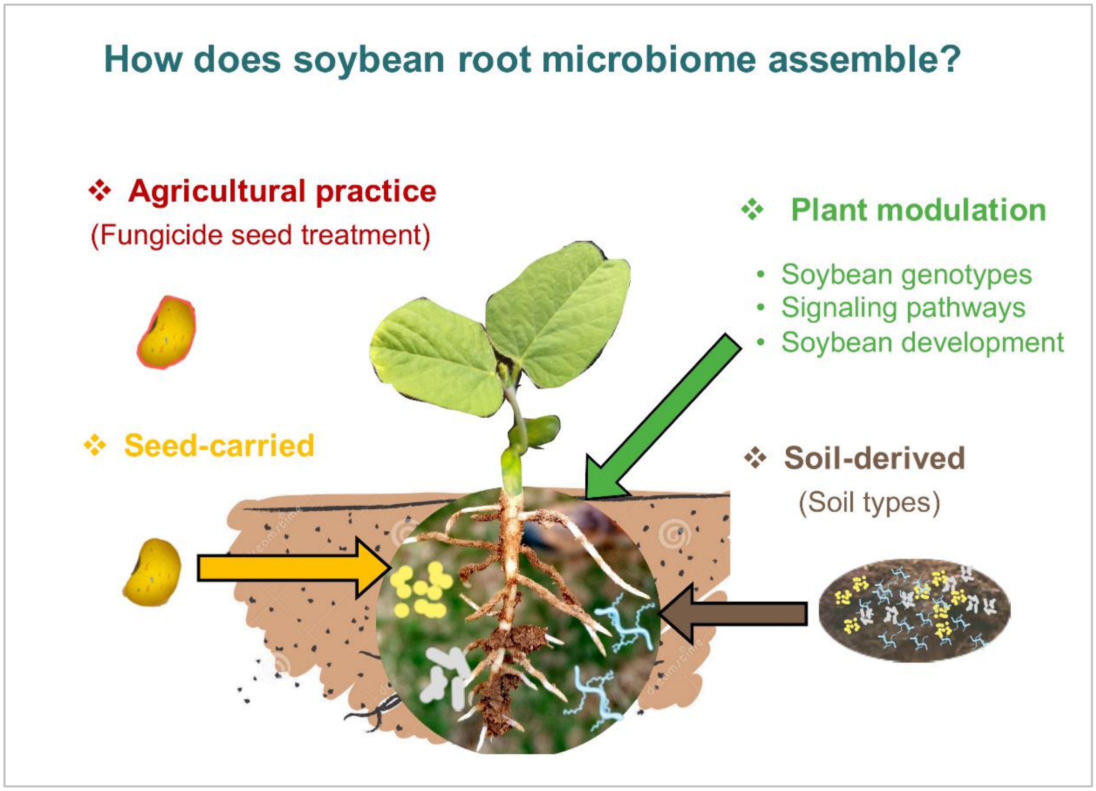

Fig. 1.1 A schematic illustration of research questions.

Chapter 2 Soil indigenous microbiome and plant genotypes cooperatively modify soybean rhizosphere microbiome assembly

Declaration

The content for this chapter has been published in BMC Microbiology as below:

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Abstract

Plants have evolved intimate interactions with soil microbes for a range of beneficial functions including nutrient acquisition, pathogen resistance and stress tolerance. Further understanding of this system is a promising way to advance sustainable agriculture by exploiting the versatile benefits offered by the plant microbiome. The rhizosphere is the interface between plant and soil, and functions as the first step of plant defense and root microbiome recruitment. It features a specialized microbial community, intensive microbe-plant and microbe-microbe interactions, and complex signal communication. To decipher the rhizosphere microbiome assembly of soybean (*Glycine max*), we comprehensively characterized the soybean rhizosphere microbial community using 16S rRNA gene sequencing and evaluated the structuring influence from both host genotype and soil source. Comparison of the soybean rhizosphere to bulk soil revealed significantly different microbiome composition, microbe-microbe interactions and metabolic capacity. Soil type and soybean genotype cooperatively modulated microbiome assembly with soil type predominantly shaping rhizosphere microbiome assembly while host genotype slightly tuned this recruitment process. The undomesticated progenitor species, *Glycine soja*, had higher rhizosphere diversity in both soil types tested in comparison to the domesticated soybean genotypes. *Rhizobium*, *Novosphingobium*, *Phenylobacterium*, *Streptomyces*, *Nocardioides, etc.* were robustly enriched in soybean rhizosphere irrespective of the soil tested. Co-occurrence network analysis revealed dominant soil type effects and genotype specific preferences for key microbe-microbe interactions. Functional prediction results demonstrated converged metabolic capacity in the soybean rhizosphere between soil

types and among genotypes, with pathways related to xenobiotic degradation, plant-microbe interactions and nutrient transport being greatly enriched in the rhizosphere. This comprehensive comparison of the soybean microbiome between soil types and genotypes expands our understanding of rhizosphere microbes assembly in general and provides foundational information for soybean as a legume crop for this assembly process. The cooperative modulating role of the soil type and host genotype emphasizes the importance of integrated consideration of soil condition and plant genetic variability for future development and application of synthetic microbiomes. Additionally, the detection of the tuning role by soybean genotype in rhizosphere microbiome assembly provides a promising way for future breeding programs to integrate host traits participating in beneficial microbiota assembly.

2.1 Introduction

It has been widely recognized that plants utilize associated microbes for a range of beneficial functions including nutrient acquisition, pathogen resistance and stress tolerance (Lucas William Mendes et al. 2018). Recent studies consistently demonstrate that the plant microbiome greatly extends plants' adaptations to changing environments (Bakker et al. 2018; Goh et al. 2013). These results suggest a promising new avenue of research for sustainable agriculture (Pieterse, de Jonge, and Berendsen 2016). Further, microbe community assembly is not static or passive; plants can actively modulate the assembly of their beneficial microbiome in response to stressors (e.g., drought and pathogen infection). This dynamic response further highlights the possibility of optimizing crop yields by exploiting beneficial plant-microbe interactions (Bakker et al. 2018; Schlaeppi and Bulgarelli 2015; Berendsen, Pieterse, and Bakker 2012).

The rhizosphere is an interface between plant root and soil characterized by a dynamic microbial community with intensive microbe-microbe and plant-microbe communication mediated by plant molecular signals, especially secondary metabolites (Philippot et al. 2013). At this root-microbe interface, plants and microbes have evolved intimate interactions. Plants allocate a significant portion of photosynthates as root exudates that serve as resources for microbes, and in return, microbes help to increase plant fitness via various plant growth promoting impacts (Pieterse, de Jonge, and Berendsen 2016; Dutta and Podile 2010). The rhizosphere is also the first line of plant defense to pathogen infection (Lucas William Mendes et al. 2018) and acts as the initial filter for the subset of microbes that will colonize the root as endophytes (Lundberg et al. 2012). Understanding the major factors that shape the rhizosphere microbiome assembly and the mechanisms of mutual adaptation between microbes and plants in response to changing environmental conditions will help to identify potential targets for future crop breeding and management.

Comprehensive characterization and comparison of rhizosphere microbiomes among numerous plant species under different conditions has consistently revealed the crucial impacts of soil source (Peiffer et al. 2013; Lundberg et al. 2012) and plant genetic traits (Fitzpatrick et al. 2018; Lu et al. 2018; Turner et al. 2013) on rhizosphere microbiome assembly. The pool of microbes available in the soil determines the initial microbial repertoire for this assembly process (Philippot et al. 2013). In addition, soil physio-chemical characteristics directly modulate microbial communities and may also indirectly alter rhizosphere microbiome assembly through impacts on host plant physiology (Philippot et al. 2013). Plant physiology and genetics also control rhizosphere composition. Differences in root morphology and in the quantity and quality of rhizodeposits could greatly diversify the composition and activity of the rhizosphere microbiome in a species-specific way (Philippot et al. 2013). With the advantage of nitrogen fixation by rhizobia, the root exudates of legumes differ from non-legumes in both quantity and quality, with higher exudation amounts and lower carbon-to-nitrogen ratios (Warembourg, Roumet, and Lafont 2003). This special trait of legumes may shape the rhizosphere microbiome assembly differently compared with non-legume plants. Turner et al. (2013) compared rhizosphere microbiomes between wheat, oat, and pea and found a higher rhizosphere effect (i.e, compositional and functional difference of microbiome between rhizosphere and nearby soil) in pea compared with the cereals. In addition to soil source and plant genetic traits, domestication, soil nutrient status and abiotic stress mediate rhizosphere microbiome assembly to different degrees (Bulgarelli et al. 2015; Timm et al. 2018; Fitzpatrick et al. 2018; Castrillo et al. 2017).

The impact of plant genotypes on rhizosphere microbiome composition is usually reported to be very weak but varies depending on soil context and plant species studied (Pérez-Jaramillo, Mendes, and Raaijmakers 2016). For example, the composition of rice root microbiome was significantly influenced by rice genotype when grown under controlled greenhouse conditions, whereas no impact was detected under field conditions (Edwards et al. 2015). Peiffer et al. (2013) suggested a small but significant impact of maize genetic variations on bacterial diversity under field conditions by a comprehensive comparison across 27 inbred lines. A comparison of the rhizosphere microbiome between barley genotypes with different domestication histories also revealed small but significant impacts, and these genotype-dependent impacts were manifested by differing the abundance of a few specific taxa instead of whole community-level differences (Bulgarelli et al. 2015). Although genotype level modification of microbial composition appears to be modest, genes participating in immune response, nutrient response, and stress response could change the abundance of specific microbial consortia, which in turn would profoundly alter host performance (Castrillo et al. 2017; Panke-Buisse et al. 2015; Timm et al. 2018; Lebeis et al. 2015). One example of this change was reported by Hanley et al. (2015), in which genotype differences in the ability to associate with *Pseudomonas fluorescens* between wild *Arabidopsis* accessions were found to be related to host fitness (Haney et al. 2015).

Soybean is an important crop worldwide as an essential food resource for protein and vegetable oil and also is the largest feedstock source for biodiesel production in the

United States (G. L. Hartman, West, and Herman 2011; Hill et al. 2006; Herridge, Peoples, and Boddey 2008; “Monthly Biodiesel Production Report - Energy Information Administration” n.d.). Soybeans form a symbiotic relationship with the nitrogen-fixing rhizobia. As improvements of the nitrogen-fixing capacity of soybeans is a major research goal, numerous studies have been conducted to understand the process and signaling pathways that mediated this symbiotic interaction. Soil physico-chemical characteristics, including soil moisture, temperature, pH and nutrient status, have consistently been reported as crucial factors determining the efficiency of nodulation and nitrogen fixation (Vance 2001; Zahran 1999; Salvagiotti et al. 2008). Due to this predominant symbiotic interaction between rhizobia and soybean, the microbiome composition of soybean may differ from non-legume plants. This difference was observed in the root microbiome of another legume, *Trifolium pratense,* in which rhizobia accounted for 70% of the whole root microbiome (K. Hartman et al. 2017).

To evaluate the relative contribution of soil source and host genetic traits in rhizosphere microbiome assembly, six soybean genotypes with varying traits and two soil types with distinct microbiome compositions were chosen to compare rhizosphere microbiome assembly both compositionally and functionally. Considering the distinct developmental traits of the genotypes and distinguished microbiome differences between soil types, we hypothesize that both factors will significantly and cooperatively manipulate the structure and composition of rhizospheric microbiota. It has been recognized that microbe-microbe interaction is another crucial driving force for rhizosphere microbiome assembly (Bulgarelli et al. 2015; Hassani, Durán, and Hacquard 2018). To examine this factor, we also compared the difference of microbial network patterns between bulk soil and rhizosphere and among genotypes in terms of the network complexity, modularities, and key taxa. By integrating the information from differential abundance analysis, microbial network, and metabolic pathway results, we aim to establish a foundation of knowledge about how the soybean rhizosphere is structured.

2.2 Methods and materials

In this study, five soybean genotypes with unique ecological or physiological traits were selected to evaluate genotype impacts on rhizosphere microbiome assembly (Table 1), including cv. Williams (WIL), a drought-tolerant cultivar (DRT), a cyst nematode-resistant line (CNR), a non-nodulating mutant of Williams (NNW), and cv. Williams 82 (W82). An accession of the undomesticated progenitor species of soybeans, *Glycine soja* (SOJ), was also included. The seeds were provided by the USDA, Agricultural Research Service, Germplasm Resources Information Network (GRIN). All soybean seeds were surface sterilized with a 10% sodium hypochlorite solution for 30 mins, followed by three rinses with deionized distilled water. Seeds were germinated on paper in a 26°C incubator in darkness for two days. Germinated soybean seeds were transplanted to autoclaved vermiculite. Just before the soybeans reached trifoliolate stage (about 11 days after germination), fresh agriculture soil of pH around 7.5 was collected from a depth of 20cm from the East Tennessee AgResearch and Education Center Plant Science Unit. Fresh forest soil was obtained from the University of Tennessee Plateau Research and Education Center, with a soil pH of about 4.8. After field collection, all fresh soils were transported to the greenhouse the same day after collection. After removal of roots and debris, soil was homogenized by mixing, then allocated to pots (diameter=20cm). The second day after soil collection, soybean seedlings at the trifoliolate stage were transplanted into the fresh soil and grown in the greenhouse until flowering stage (30°C day/20°C night, 16h light/8h dark, relative humidity of 60-80%). Fifteen pots of soil without soybeans were used as bulk soil control. Each treatment group (genotype by soil) was started with 10 biological replicates. Both soybean seedlings and control pots were watered as needed every other day.

At the flowering stage, soybean rhizosphere soil samples were collected according to Lundberg et al. (2012). Briefly, the root ball of soybeans was gently removed from the pot and soil loosely attached to the roots was removed by mild shaking. Soybean roots with tightly attached soil were put into a 50-mL centrifuge tube filled with 30 mL of autoclaved phosphate buffer (per liter: 6.33 g of $NaH_2PO_4.H2O$, 16.5 g of $Na_2HPO_4.7H2O$, 200 µL Silwet L-77). The tube was vortexed at maximum speed for 30 s and the slurry was filtered through a 100-µm cell strainer into a new 50-mL centrifuge tube. The soil slurry was then centrifuged to precipitate soil particles. After another round of resuspension and centrifuging, the soil pellet was collected into 1.5 mL eppendorf tubes. To eliminate the interference of the soil crust on microbiome characterization, the surface soil was removed from the control pot and the remaining soil was well homogenized. A similar amount of soil as that of rhizosphere was collected from the soil mix and defined as bulk soil. All of the extracted soil samples were flash frozen in liquid nitrogen and stored at -80°C before DNA extraction.

Soil DNA was extracted with the *MoBio* soil DNA extraction kit following the manufacturer's protocol. Most of the samples yielded concentrations of about 200 ng/µL. 16S rRNA gene based bacteria profiling were accomplished with MiSeq 275 bp paired-end sequencing targeted V3-V4 regions, with forward primer 341F= 5′-CCTACGGGNGGCWGCAG-3′ and reverse primer 785R = 5′-GACTACHVGGGTATCTAATCC-3′ (Takahashi et al. 2014). Library preparation followed the Illumina 16S metagenomic sequencing protocol. Briefly, for the first step PCR, 16S rRNA gene specific primer with adapter overhangs was used to amplify template out of genomic DNA utilizing 2X KAPA HiFi HotStart ReadyMix with the following PCR cycle: 95°C for 3 minutes; 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes, then hold at 4°C. During the second step of PCR, dual indices and Illumina sequencing adapters were attached to the template amplified from step one using the Nextera XT Index Kit with PCR cycle: 95°C for 3 minutes; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes and hold at 4°C. To eliminate the amplification of chloroplast and mitochondria sequences from any plant contamination, peptide nucleic acid (PNA), including anti-mitochondrial PNA (mPNA) 5′-GGCAAGTGTTCTTCGGA-3′ and the anti-plastid PNA (pPNA) 5′-GGCTCAACCCTGGACAG-3′ were used to block their elongation during the first step of PCR (Lundberg et al. 2013).

Mothur software was used to process 16S rRNA gene sequences, including quality control, assembly, alignment, chimera removal, *SILVA*-based OTU clustering at 97 percent similarity, and naive Bayesian classifier-based OTU classification against Ribosomal Database Project (RDP) training set (Kozich et al. 2013). During this process, any sequence pairs that have a mismatch within the primer region were removed before assembly. Chimera sequences were detected and removed using the mothur-incorporated vsearch tool based on the UCHIME algorithm (Rognes et al. 2016; Edgar et al. 2011). Sequences that belong to chloroplast, mitochondria, eukaryotes, and archaea were discarded before OTU clustering. To alleviate the bias introduced by uneven sequencing depth, rarefaction at the minimum sample sequencing depth (19023) was used for normalization before subsequent microbial community analysis in R.

Beta diversity between samples was calculated with the Bray-Curtis weighted distance, and principal coordinate analysis (PCoA) using this dissimilarity matrix were applied to visualize the differences between microbial communities between treatments. Permutational multivariate analysis of variance (PERMANOVA) was used to evaluate the marginal effects contributed by each factor to the distinct microbial composition pattern between treatments using 999 permutations. In addition to PERMANOVA, partial canonical analysis of principal coordinates (CAP) (Clarke 1993) based on Bray-Curtis distance was used to further evaluate the impacts of genotypes on rhizosphere microbiome assembly and visualized through a CAP plot. Considering the strong similarity of bacterial composition between fresh soil samples (before greenhouse experiment) and bulk samples (after greenhouse experiment), subsequent *LefSe*, network and KEGG pathway analysis were performed on combined bulk soil and fresh soil samples (hereafter were represented as soil treatment).

Differential abundance analysis of bacteria at different taxa levels between treatments were performed with *LefSe* under one-against-all mode (i.e., one taxa is considered to be significantly different only when it is significantly different against all remaining treatments) (Segata et al. 2011). The LDA logarithmic score was calculated with 200 bootstraps iterations, and any taxa with α less than 0.05 were defined to be significantly different between treatments. For the overall abundance comparison between soil and rhizosphere across all bacterial taxa levels, the LDA logarithmic score threshold was set to 4.0. To provide a comprehensive comparison of bacteria enrichment and depletion in soybean rhizosphere across all treatments, *LefSe* analysis between each pair of rhizosphere and soil samples were performed at the genus level. To improve the accuracy and robustness of the differential abundance analysis, any genus with a total count smaller than 50 was removed before LefSe analysis. Under one-against-all comparison mode, each genus with an α less than 0.05 and an LDA score greater than 2 was defined to be significantly different between rhizosphere and soil. Significantly enriched and depleted genera together with their LDA scores across treatments were merged to generate a tree file and an annotation file for GraphlAn visualization (Asnicar et al. 2015). Any genus that was significantly enriched or depleted in the rhizosphere were annotated with red or blue colors respectively, while yellow color indicated no significant difference between rhizosphere and soil.

To infer the difference of microbe-microbe interaction patterns between soil types and among genotypes, samples were grouped based on treatments, i.e., Ag_Soil, Ag_WIL, Ag_DRT, Ag_CNR, Ag_NNW, Ag_SOJ, Ag_W82, For_Soil, For_WIL, For_DRT, For_CNR, For_NNW, For_SOJ and For_W82 (Ag for agricultural soil, For for forest soil, genotype abbreviations as defined in Table 2.1). To infer robust microbe-microbe interactions, any OTU with a total count smaller than 10 were removed to eliminate the confounding impacts introduced by these rare taxa. A co-occurrence correlation network between OTUs was calculated with *SparCC* algorithm with 20 interactions (Friedman and Alm 2012). Corresponding p-values for each correlation were determined based on 200 iterations of the bootstrapping process. During the bootstrapping process, 200 sets of simulated count matrices were generated from the original count matrix. By comparing the SparCC correlation matrix generated using simulated datasets and that of the original dataset, p-values were calculated. For overall network topological traits comparison, each edge with a p-value less than 0.01 were kept for visualization. Further simplification of the networks was done by selecting the top 50 nodes with the largest connection degrees. The integrated network comprising all treatments was generated by uniting individual networks based on shared nodes, with different edge colors representing different treatments and different vertex colors depicting bacterial OTU. The network visualization and topological properties measurements were done with the R package igraph (Csardi and Nepusz 2006).

To investigate the difference of potential ecological functions between bulk soil and rhizosphere microbiomes across all treatments, the R package *Tax4Fun* was used to predict microbial functional and metabolic capacities by linking 16S rRNA gene-based taxonomic profiles to pre-calculated KEGG references (Aßhauer et al. 2015). The predicted normalized KEGG pathway output was then used to investigate the enrichment of microbial pathways between soil and rhizosphere by DESeq2 (Love, Huber, and Anders 2014). Pathways with an adjusted p-value less than 0.01 and related to plant microbiome functions were selected for subsequent visualization in a heatmap using the *pheatmap* R package (Kolde 2015).

2.3 Results

A total of 19358039 raw reads fro--m 136 samples were generated after paired-end sequencing with a read length of 275 bp. Quality analysis with FastQC suggested that the first 200-250 bp of each read had a quality score higher than 30 (Fig. S2.1), and 88-95% of the sequences had an exact match in the primer region. After several steps of stringent trimming and filtering of chimeric and non-bacterial sequences, 9945986 reads were clustered into 175957 OTUs based on a threshold of 97% sequence similarity. Most of the samples yielded about 50000 reads, with the minimum sequencing depth of 19023 and the maximum depth of 247930 (Fig. S2.2). The rarefaction curve suggested consistent bacterial OTU richness across samples, with no obvious outlier samples (Fig. S2.3). After rarefaction to the minimum sequencing depth, 76864 OTUs remained in the 136 samples, belonging to 25 phyla, 99 classes, 122 orders, 244 families and 642 genera.

2.3.1 Soybean rhizosphere demonstrates different but dependent microbial community composition compared to bulk soil

Overall, the microbial community of the soybean rhizosphere microbiome is significantly different from that of the surrounding bulk soil, with some taxa being consistently recruited to the rhizosphere regardless of the soil type. However, some other bacterial taxa were specifically enriched in soybean rhizosphere in a soil-dependent way.

2.3.1.1 Phylum, class, order and family level comparison

At the phylum level, bacterial communities were dominated by *Proteobacteria*, *Acidobacteria*, *Actinobacteria,* and *Bacteroidetes* in both agricultural and forest soils, with the next most abundant phyla being *Firmicutes* in agriculture soil, and *Verrucomicrobia* and *Planctomycetes* in forest soil (Fig. 2.1). The composition of microbes immediately after collection (fresh soil) and after 2 months in the greenhouse (bulk soil) were similar, indicating that the greenhouse environment and the time lapse did not largely alter microbial communities. Comparison of bulk and fresh soil samples to rhizosphere samples revealed much greater differences. Differential abundance analysis results indicated that *Proteobacteria*, *Actinomycetales* and *Enterobacteriaceae* were significantly enriched from bulk soil to rhizosphere in both soil types across all the six genotypes, while *Acidobacteria* and *Verrucomicrobia* were consistently depleted in soybean rhizosphere (Fig. 2.2). However, the enrichment/depletion pattern of bacterial phyla in the soybean rhizosphere was not entirely consistent between soil types; *Firmicutes* (especially *Bacilli*) was preferably enriched in the rhizosphere when grown in agriculture soil, while *Bacteroidetes* (specifically *Chitinophagaceae*) were selectively accumulated when growing in forest soil. Similarly, *Alphaproteobacteria* (especially *Rhizobiales*) and *Betaproteobacteria* (specifically *Burkholderiales*) were discriminantly enriched in agriculture and forest soil respectively. Although *Gammaproteobacteria* was consistently enriched in the rhizosphere across all treatments, the enrichment of bacteria within the *Gammaproteobacteria* class also differed between soil types, with *Xanthomonadaceae* preferably enriched in forest soil while *Pseudomonadaceae* were preferably recruited when grown in agriculture soil. This divergent enrichment/depletion pattern in soybean rhizosphere between soil types indicates the dominant impacts of the soil sources and their starting microbial pools on rhizosphere microbiome assembly.

2.3.1.2 Genus level

To provide a more detailed understanding of bacteria assembly in soybean rhizosphere under different soil conditions and host genetic background, *LefSe* analysis was conducted at the genus level to determine the enrichment/depletion pattern between each pair of rhizosphere and soil samples (e.g., Ag_WIL rhizosphere vs. soil samples) with an LDA score threshold of 2. In total, the relative abundances of 299 out of 642 bacterial genera were detected to be significantly different between rhizosphere and soil samples. Among these 299 genera, 11 were consistently enriched in the soybean rhizosphere for both soil types across the six genotypes: *Rhizobium*, *Novosphingobium*, *Phenylobacterium*, *Streptomyces*, *Nocardioides*, *Nocardia*, *Amycolatopsis*, *Dyadobacter*, *TM7_genus_incertae_sedis*, *Sphingobacteriaceae_unclassified*, and *Enterobacteriaceae_unclassified*. In contrast, 113 out of the 299 genera (*Gp15*, *Gp13*, *Gp9*, *Gp6*, *Gemmata*, *Rhodospirillales-unclassified*, *Betaproteobacteria-unclassified*, *Rhodocyclaceae-unclassified*, *Deltaproteobacteria-unclassified*, *Planctomycetaceae-unclassified*, and *Bacteria-unclassified*) were steadily depleted in the rhizosphere (Fig. 2.3).

Consistent with phylum level results, numerous bacterial genera were selectively enriched/depleted in the rhizosphere when grown in one soil type instead of the other. For example, *Bradyrhizobium*, *Pseudoxanthomonas*, *Kribbella*, and *Agromyces*, etc. were favorably accumulated in the soybean rhizosphere when grown in agriculture soil. Meanwhile, *Burkholderia*, *Rudaea*, *Dyella* and *Mucilaginibacter*, etc. were discriminatively recruited to the soybean rhizosphere when grown in forest soil. Likewise, *Gp1* and *Pasteruria* were significantly decreased in the soybean rhizosphere when grown in agriculture soil while *Gp2* was selectively depleted when grown in forest soil. In total, 37 genera were specifically enriched in the rhizosphere when soybeans were grown in agriculture soil while 13 genera were specifically enriched in forest soil (Table S2.2). Among the 37 specifically enriched genera, only one genus was absent in the soybean rhizosphere when growing in forest soil, while none out of the 13 genera was absent in soybean rhizosphere when grown in agriculture soil. In other words, this soil-type specific bacteria enrichment may be attributed to differences in rhizosphere assembly processes instead of absence of a specific taxon in the microbial pool. Additionally, even among those that were consistently enriched, the degree of enrichment also varied and depended on the soil type. For example, differential abundance analysis indicated that *Rhizobium*, *Streptomyces* and *Novosphingobium* were constantly enriched in soybean rhizosphere across all genotypes and soil types. However, the degree of this enrichment was more dominant when grown in agriculture soil compared with that of forest soil (Fig. 2.4). In contrast, the depletion of *Acidobacteria* was more distinct in soybean rhizosphere when the plants were grown in forest soil in comparison with those grown in agriculture soil.

In addition to soil type effects, between-genotype differences in bacteria enrichment/depletion patterns were also apparent (Fig. 2.3). As visualized in the bacterial genus abundance boxplots, *Pseudomonas* and *Stenotrophomonas* were enriched in all soybean genotypes except *Glycine soja* (Fig. 2.4). Similarly, the recruitment of *Rhizobium*, *Pantoea* and *Mucilaginibacter* in *Glycine soja* was also limited compared with the other five genotypes. However, the recruitment of *Streptomyces* and *Kribbella* was more evident in the wild species accession (SOJ) compared with other genotypes when grown in agricultural soil. Compared with other genotypes, non-nodulating soybeans (NND) were less attractive to *Novosphingobium* as demonstrated by its lower abundance in soybean rhizosphere.

2.3.2 Dominant impacts of soil indigenous microbe pool and soil environment on rhizosphere microbial community composition

To quantify the differences in microbial community composition between samples, Bray-Curtis dissimilarity was calculated and visualized in a PCoA plot. The separation pattern between samples indicated distinct microbial community composition between the rhizosphere and bulk soil as well as between soil types (Fig. 2.5). The first two axes explained more than 70% of microbial community variance between samples, with samples clearly separated by soil type on the first axis (64.6% explained variance), while compartment (rhizosphere or bulk soil) was primarily represented along the second axis (7.1% explained variance).

To evaluate the relative impacts of soil type and compartment on microbial community composition, a PERMANOVA was used to partition the source of variance. Here, the compartment impacts were referred to microbiome differences between soil samples and soybean rhizosphere samples. The results suggested that soil type is the most dominant explanatory factor for the distinct microbial community structure between samples, explaining 62% of the overall variance of the microbe composition across all samples (PERMANOVA marginal effects: $F_{(1,131)} = 259.65$, $p < 0.001$). Larger soil type effects for bulk and fresh soil microbial communities (81.37% variance) were detected compared with the rhizosphere microbiome (70.61%). Compartment effects were the secondary key factor (explaining 6% of variance) that contributed to the overall divergent microbial community (PERMANOVA marginal effects: $F_{(2,131)} = 12.86$, $p < 0.001$). The compartment effects are in fact mainly contributed by the rhizosphere, considering the very similar microbiome composition between fresh and bulk soil samples. Thus, compartment effects will be referred to as rhizosphere effects hereafter. Within each individual soil type, the rhizosphere effects were more evident, with 28.16% (PEMANOVA marginal effects: $F_{(2,65)} = 12.74$, $p < 0.001$) and 38.48% (PERMANOVA marginal effects: $F_{(2,65)} = 20.33$, $p < 0.001$) variance of microbiome composition being explained in agriculture and forest soil correspondingly. A significant interaction of soil type and rhizosphere effects was also detected for the overall microbiome composition (PERMANOVA marginal effects: $F_{(2,129)} = 12.67$, $p < 0.001$). The impact of sequencing depth on microbe composition results was evaluated and found to be nonsignificant when soil type and compartment were taken into account altogether (PERMANOVA marginal effects: $F_{(1,131)} = 1.815$, $p = 0.138$).

2.3.3 Soybean genotype slightly tunes soybean rhizosphere microbiome assembly

To evaluate the impacts of soybean genotype on rhizosphere microbiota assembly, the dataset was subdivided into two subsets composed of agriculture and forest rhizosphere samples. A PERMANOVA test indicated significant impacts of the soybean genotype in both agriculture (PERMANOVA marginal effects, $F_{(5,45)} = 2.70$, $p<0.01$) and forest (PERMANOVA marginal effects, $F_{(5,45)} = 2.44$, $p < 0.01$) rhizosphere microbe composition, with 23.08% and 21.32% variance explained respectively. The differences driven by genotypes were not evident when visualized using an unconstrained ordination method, i.e., PCoA (Fig. 2.6A and 6B). However, when illustrated using canonical analysis of principal coordinates (CAP), the influence of microbe community compositions due to genotypes is clearer (Fig. 2.6C and D). CAP analysis is a good option when effects are not easily detected by unconstrained ordination, as it can utilize treatment information (Anderson and Willis 2003). Genotype impacts were more evident for soybeans grown in agriculture soil, with the drought-resistant genotype (DRT) and wild-type genotype (SOJ) more divergent from others (Fig. 2.6C and 6D). In contrast, the bacterial community structure of Williams (WIL), Williams non-nodulating mutant (NNW) and Williams 82 (W82), all of which share the Williams genetic background, were more similar and had no clear separation pattern on the CAP plot. Significant interactive impacts of soil type and genotype were detected in determining soybean rhizosphere microbiome composition (PERMANOVA marginal effects: $F_{(5,89)}$=2.03, p=0.04).

Another important aspect of variability worth examining is flowering time. All rhizosphere samples were taken as soon as plants reached the flowering stage, in order to mitigate the impact of different developmental stages that might impact the results. However, as the six genotypes are from different maturity groups (i.e. are adapted to different climatic zones), the individual soybeans in this study flowered at different times over the course of six weeks. A PERMANOVA test suggested significant impacts of flowering time on both agriculture and forest rhizosphere microbe composition. After partialling out flowering time as a factor, the soybean genotype still explained 3% of the variance (capscale, $F_{(1,39)}$=2.29, p<0.01). Due to the high correlation between flowering time and genotypes, it is difficult to rule out the pure genotype effects on rhizosphere microbiome assembly from that of flowering time when tested using all samples. To help evaluate the soybean genotype impacts, we grouped samples that flowered on the same date and visualized their rhizosphere microbiome composition with a PCoA plot (Fig. S2.4). We observed distinct rhizosphere microbiome composition between Williams (WIL) and the non-nodulating mutant of Williams (NNW). These two genotypes are genetically identical other than a mutation of gene $R_{j5,6}$, which is a receptor gene of rhizobia nodulation factor (Hayashi et al. 2012). The divergent rhizosphere microbiomes between these two genotypes indicate that their genetic difference indeed confers direct impact on rhizosphere composition independent of flowering time differences.

2.3.4 Significant rhizosphere effects on microbiome diversity and microbe-microbe interactions

Indigenous microbial community diversity was significantly higher in agriculture soil than forest soil, which held true for both bulk soil and the soybean rhizosphere ($F_{(1,130)}$=228.82, p<2.20e-16) (Fig. 2.7). A significant rhizosphere effect was reflected by reduced microbiome diversity in soybean rhizosphere compared with that of fresh and bulk soil samples ($F_{(2,130)}$=23.96, p=1.39e-09), with no significant difference detected between the latter two. Rhizosphere microbiome diversity also differed significantly between genotypes in both agriculture (ANOVA; $F_{(5,45)}$ =9.46, p=3.22e-06) and forest soil (ANOVA; $F_{(5,45)}$ =4.99, p=0.10e-02). The diversity of the drought-tolerant genotype (DRT) was significantly and consistently smaller than other genotypes in both soil types. In addition, there was a significant interaction effect of soil type and genotypes on rhizosphere microbiome diversity ($F_{(5,90)}$=4.42, p=0.12e-02).

Beyond the direct modulation by soil and plant host, the interactions between microbes act as another selective force for root microbiome assembly (Hassani, Durán, and Hacquard 2018). To elucidate these interactions in the rhizosphere and infer key microbial consortia, we characterized co-occurrence correlation networks between microbes and compared the difference of those interaction patterns between treatments. Overall, microbe-microbe interactions in soil were denser and more connected compared with that of rhizosphere as indicated by higher edge density and average connection degree in soil samples (Table 2.2), which is consistent with the reduced bacterial diversity in the rhizosphere. The complexity of the microbial network in WIL was consistently higher than the other five genotypes in both soil types. However, there were no strong correlations between network complexity and microbial diversity when fitted using linear regression between average network density and Shannon diversity (Fig. S2.5).

When all of the significant microbe-microbe interactions were taken into account at α<0.01, there was no significant separation of the rhizosphere microbiome networks between soybean genotypes, but the difference between soil types was distinguishable (Fig. S2.6). The connection degree of each node varied between 1 and 337, with the top 25 most connected OTUs belonging to *Mycobacterium*, *Sphingomonas*, *Massilia*, *Bradyrhizobium*, *Bacillus*, *Gp16*, *Streptomyces*, *Phenylobacterium*, *Rhizobium* and *TM_genus_incertae_sedis* genera. A high percentage of nodes were shared between soil and rhizosphere networks, with 64-72% of nodes being shared in the two compartments in agriculture soil, while 71-75% overlap between compartments was detected in forest soil. The positive correlation ratios (the positive microbe-microbe correlations out of all significant interactions) were detected to be higher in the soybean rhizosphere compared with soil samples. To evaluate the correlation of taxa abundance and its connection densities, linear regression models were fitted using OTU relative abundance and corresponding node degree (Fig. S2.7). The results showed weak but significant correlation between OTU abundance and corresponding node degree. Several OTUs with high abundance showed limited interactions with other taxa, including OTU000004 and OTU000012, belonging to *Burkholderia* and *Rhizobium* respectively. In contrast, several rare taxa such as OTU000159 and OTU000349, belonging to *Mycobacterium* and *Spartobacteria_genera_incertae_sedis* showed a high degree of connections with other bacteria.

To simplify the network and identify key microbe-microbe interactions, the top 50 OTUs with the highest connection degrees were selected from each treatment for detailed comparison. Within this subset, the network complexity of soil samples was still consistently higher than that of the rhizosphere (Table 2.2). The network of WIL was denser compared with other genotypes in both soil types. However, the network pattern of the other five genotypes, such as network density and positive correlation ratio, varied between soil types (Fig. S2.8). When grown in agricultural soil, DRT, SOJ and W82 had higher positive interactions than other genotypes whereas CNR, NNW and W82 had with higher positive interactions when growing in forest soils. These results again confirm the cooperative modulating role of soybean genotypes and indigenous soil types in microbe-microbe interactions.

To understand the overall network patterns between treatments, the individual top 50 networks were united to a comprehensive network based on shared OTUs between treatments (Fig. 2.8). After the union process, the number of nodes was reduced from 700 to 566, with most belonging to *Proteobacteria* (105), *Bacteria_unclassified* (95), *Acidobacteria* (91), *Planctomycetes* (55), *Actinobacteria* (54), *Verrucomicrobia* (51) and *Bacteroidetes* (47). OTUs with the highest number of connections with others belonged to *Bradyrhizobium*, *Mycobacterium*, *Sphingomonas*, *Gp4*, *Spartobacteria_genera_incertae_sedis*, *TM7_genus_incertae_sedis*, *Massilia* and *Gp16*. The differences in microbe-microbe interactions between soil types and among genotypes were exemplified by the high modularity of subnetworks between soybean genotypes, which was strikingly different than the analysis that included all significant correlations. In contrast to the large percentage of shared OTUs between treatments when all significant OTUs were taken into account, only a few OTUs were shared between soil and rhizosphere as well as among genotypes when the top 50 key microbes were concerned. These shared OTUs function as connectors between the subnetworks (Fig. 2.8) and are classified in the genera *Bacillus*, *Streptomyces*, *Bradyrhizobium*, *Rhizobiales_unclassified*, *Arthrobacter*, *Caldilineal*, *Mycobacterium*, and *Gp1* as well as several unclassified genera in the phylum of *Verrucomicrobia*. Such bacterial consortia may play a dominant and persistent role in modulating microbial community composition via prevalent interactions with other bacteria.

2.3.5 Specialized microbiome function in soybean rhizosphere

Genotype-specific rhizosphere effects were detected in the soybean rhizosphere microbiome as reflected by differential microbial community compositions between rhizosphere and bulk soil as well as among genotypes. To understand the functional differences of these communities, we predicted the potential metabolic capacities of both the soil and rhizosphere microbiomes using *Tax4Fun*. The results indicated divergent metabolic capacities between soybean rhizosphere microbiota and bulk soil community (Fig. 2.9). Of particular interest, the enrichment/depletion of metabolic pathways was consistent between soil types and across genotypes regardless of the divergent bacteria composition.

Metabolic pathways related to biodegradation of xenobiotics, including glutathione metabolism, geraniol degradation, limonene and pinene degradation as well as naphthalene degradation, were significantly and consistently enriched in the soybean rhizosphere regardless of soil types. Pathways involved in nutrient transformation and transport, such as phosphotransferase systems and ABC transporters were also enriched in soybean rhizosphere. In addition, bacterial functions related to plant-microbe interactions were also enriched in the rhizosphere, such as flagella assembly, bacterial secretion system, and biosynthesis of siderophore. In contrast, metabolic pathways involved in antibiotic production, including streptomycin biosynthesis and biosynthesis of ansamycins, were enriched in the bulk soil environment. The metabolic pathways for fructose, mannose, starch and sucrose metabolism were accumulated in soil as well. Another functional group significantly expanded in soil bacteria involved DNA repair and recombination including nucleotide excision repair and homologous recombination.

2.4 Discussion

2.4.1 Soil type-dependent rhizosphere effects

In our study, *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Actinobacteria* were the most dominant bacterial phyla in soybean rhizosphere, which is consistent with previous reports about the soybean rhizosphere microbiome (Lucas W. Mendes et al. 2014;

Sugiyama et al. 2014; Xiao et al. 2017). *Gammaproteobacteria* and *Actinomycetales* were consistently enriched in the soybean rhizosphere in both soil types, which is consistent with the thought that *Actinobacteria* and *Proteobacteria* as copiotrophs are more competitive in a nutrient-enriched environment like rhizosphere, while oligotrophs like *Acidobacteria* and *Verrucomicrobia* are more abundant in soil with poor nutrients (Pérez-Jaramillo, Mendes, and Raaijmakers 2016). However, at the genus level, this enrichment exhibited differences for some specific bacteria genera within *Gammaproteobacteria* and *Actinomycetales*, which greatly depended on soil types and soybean genotypes. This result indicates that analysis based on different taxonomic levels may achieve inconsistent conclusions about the robustness of rhizosphere bacteria assembly. Considering the functional redundancy between different bacteria, functional analysis of rhizosphere microbiome together with compositional characterization may be more informative for understanding microbiome assembly and promoting applications for sustainable agriculture.

Rhizosphere effects on bacteria composition have been widely recognized on numerous plant species, such as maize (Peiffer et al. 2013), rice (Edwards et al. 2015), Arabidopsis *(Lundberg et al. 2012)*, poplar (Beckers et al. 2017), grapevine (Samad et al. 2017), alfalfa (Xiao et al. 2017), and sugarcane (de Souza et al. 2016). These investigations spanned monocotyledons and dicotyledons, annuals and perennials, and legumes and non-legumes. The results found differing extents of rhizosphere effects between plant species due to distinct evolution time, plant root physiology and root exudation profile between species (Fitzpatrick et al. 2018; Sasse, Martinoia, and Northen 2018). Turner et al. (2013) revealed a stronger rhizosphere effect of microbial community on pea (legume) compared with that of oat and wheat (Turner et al. 2013). Similarly, *Lotus japonicus* plants assemble a distinct rhizosphere microbial community that is influenced by root nodule symbiosis (Zgadzaj et al. 2016). In our study, strong rhizosphere effects were validated in soybean as reflected by the distinct microbial community composition and structure between rhizosphere and bulk soil. These rhizosphere effects may be influenced by the specific profile of root exudates with a high concentration of flavonoids, which are essential components of signal exchange between soybean and symbiotic rhizobia during nodule formation. The influence of root exudates was also investigated by While et al. (2015), revealing that isoflavonoids also significantly alter soybean rhizosphere bacterial diversity (White et al. 2015).

In our study, a number of well-described plant growth promoting rhizobacteria (PGPR) (Shameer and Prasad 2018), including *Rhizobium*, *Dyadobacter*, *Novosphingobium* and *Streptomyces*, were consistently enriched in soybean rhizosphere. PGPR greatly expanded host adaptations and performance by various promoting activities, including IAA and siderophore production, phosphate solubilization, and induced systemic resistance (Shameer and Prasad 2018; Gupta et al. 2015). Strong enrichment of *Streptomyces* and *Dyadobacter* was also detected in the rhizosphere of pea (Turner et al. 2013). A diverse of *Rhizobium* colonize soybean root and form nodules, providing significant benefits to the plant through nitrogen fixation. The enrichment of *Rhizobium* in the soybean rhizosphere, even in the non-nodulating soybean variety, corroborates the idea that rhizosphere recruitment may be an important first step for further selection to the rhizoplane and endosphere (Bulgarelli et al. 2013), which facilitates symbiotic interactions between bacteria and host plants.

Despite the similarities in soybean rhizosphere microbe assembly across soil types, discriminant enrichment between soil types was also revealed in our study, reflecting the dominant modulating role of the indigenous microbe pool and local soil conditions. *Bradyrhizobium*, *Kribbella*, and *Agromyces* were selectively enriched in the soybean rhizosphere when the plants were grown in agricultural soil with a neutral pH and diverse bacteria pool. In contrast, *Burkholderia* and *Mucilaginibacter* were discriminatively accumulated in the soybean rhizosphere grown in forest soil with an acidic pH and less diverse bacterial pool. *Burkholderia* has been found to be enriched near roots grown under extremely nutrient-deficient soil and function to metabolize organic acid exuded by the host to soluble phosphate (Weisskopf, Heller, and Eberl 2011; Neumann and Martinoia 2002). This result is consistent with the selective enrichment of *Burkholderia* in forest soil with lower pH. Recent research confirms that dynamic root exudates from plants can interact with microbial substrate preference to shape the rhizosphere microbiome community composition (Zhalnina et al. 2018), providing a promising avenue of research to understand the underlying mechanisms driving this selective enrichment process. Despite the predominant dependency of the soybean rhizosphere microbiome assembly on soil type, we found that the impacts of soil types on rhizosphere microbe composition was smaller in comparison to corresponding impacts on indigenous soil microbial community. This result indicates that soybean as a plant host intrinsically exerts some conserved modulating force in shaping the rhizosphere microbiome assembly. In addition, rhizosphere effects were exhibited to a higher degree when soybeans were grown in forest soil compared to those grown in agricultural soil, indicating that the degree of the rhizosphere effect differs depending on the environment. This may indicate that the plants exert variable influence on the rhizosphere microbiome depending on the environment, possibly in response to how suitable the environment is for the plant growth.

2.4.2 Soybean genotypes mildly tuning rhizosphere microbiome assembly

The modulating role of plant genotypes to rhizosphere microbiome assembly is thought to be much smaller in modern agriculture systems and domesticated crops compared with that of natural systems with a long history of coevolution (Pieterse, de Jonge, and Berendsen 2016; Philippot et al. 2013). In our study, moderate and significant tuning effects by soybean genotypes on the rhizosphere microbiome composition were detected from both the overall bacterial community level and the individual genus level. At the community level, the rhizosphere microbiome composition from Williams (WIL), Williams 82 (W82) and Williams non-nodulating mutant (NNW) were more similar, while drought resistant and wild type plants were more distinct. This corresponds to the genetic differences among the genotypes, with the Williams (WIL), Williams 82 (W82) and Williams non-nodulating mutant (NNW) all sharing the Williams genetic background. Intriguingly, this between-genotype difference was detected to be more evident when soybean genotypes were grown in agriculture soil. This soil type-dependent genotype effects again indicates the integrated regulatory role from both the soil and the plant side.

Previous work in various agricultural plant species has revealed domestication to be a profound shaping force for rhizosphere microbiome recruitment, influenced by both the reduced genetic diversity of modern genotypes and crop management practices (Pérez-Jaramillo, Mendes, and Raaijmakers 2016). Several studies revealed distinct microbial community composition in wild genotypes compared with that of modern genotypes (Pérez-Jaramillo et al. 2017; Cardinale et al. 2015; Bulgarelli et al. 2015; Zachow et al. 2014). This study also found the rhizosphere bacterial community composition of the wild accession - *Glycine soja* (SOJ) - to be different from the other modern agricultural genotypes. Specifically, the enrichment of *Rhizobium*, *Pseudomonas* and *Stenotrophomonas* in the wild soybean rhizosphere was very limited compared with the other modern genotypes. In contrast, *Streptomyces* and *Kribbella* from the *Actinobacteria* phylum were extensively recruited in the wild type. In addition, the overall bacterial diversity in the wild soybean (SOJ) rhizosphere was consistently higher in comparison to all genotypes irrespective of the soil type tested. Similarly, the study by Leff et al. (2017) revealed that wild sugar beet harbors higher bacteria diversity in its rhizosphere compared with wild type. The distinct rhizosphere microbiome recruitment of the wild accession could be a reflection of soybean trait selection along domestication. For example, root morphology changed significantly from the wild progenitor to the modern agricultural genotypes, with shallow and thick roots being preferably selected during soybean breeding history in terms of phosphorus efficiency (Zhao et al. 2004).

Soybeans benefit from a nitrogen supply provided by the nitrogen-fixing process from the symbiotic relationship with *Rhizobium* and *Bradyrhizobium* that results in a higher quality of root exudates with a lower C/N ratio (Warembourg, Roumet, and Lafont 2003). Additionally, the nitrogen fixing process alters soil physicochemical properties around root nodules, featuring a high concentration of hydrogen as a by-product of nitrogen reduction by nitrogenase (Hunt and Layzell 1993). Considering these specific traits conferred by the nitrogen fixing process, it is reasonable to expect that the bacterial community of non-nodulating genotype (NNW) would significantly differ from its nodulating isogenic line (WIL). However, no effects were detected in our study. The non-nodulating Williams mutant (NNW) selected for this study was established by silencing the $R_{j5,6}$ gene coding for GmNFR5α and GmNFR5β (*Glycine max* Nod factor receptors), which are orthologs of NFR5 receptor in *Lotus japonicus (Hayashi et al. 2012)*. As a result, this mutant exhibited neither rhizobial infection nor cortical cell division. This contrasts with previous research in *Lotus japonicus*, which found that disruption of the symbiosis pathway significantly altered rhizosphere microbial communities, even with the addition of supplemental nitrogen to soil (Zgadzaj et al. 2016). These contrasting results warrant further investigation, with possible causes including the particular genes selected to disrupt nodulation, different soil nitrogen status, or specific physiologies of the two different plant species.

2.4.3 Specialized network in rhizosphere and genotype specific preference for key microbe-microbe interactions

As a result of discriminant selection occurring in the soybean rhizosphere, the diversity of the bacterial community in the rhizosphere was significantly lower than that of soil. Consistently, microbe-microbe interactions represented by co-occurrence networks were revealed to be less complicated in the rhizosphere compared to soil, which is consistent with previous studies using shotgun metagenomics (Lucas W. Mendes et al. 2014). We found that a high abundance of a bacterial taxa is not necessarily required to be a key species in terms of microbe-microbe interactions. Rare bacteria of *Mycobacterium* were found to have a high number of interactions with other taxa, which may indicate that some rare but essential species play critical roles for community structure through dense connections with other groups (Lucas W. Mendes et al. 2014). Bacterial taxa that are consistently and highly connected with other groups potentially play key role in community structure and crucial ecological functions (Agler et al. 2016). The microbiome network identified in this study could help guide future investigations of plant-microbe interactions by focusing on hub taxa that are highly connected with other groups as well as connector taxa that provide links between modules (Poudel et al. 2016). When represented using all significant correlations, the microbial networks were quite similar between rhizosphere and soil community as well as among different genotypes. However, after reduction of network complexity by selecting the top 50 taxa, we found that soybean-genotype-featured unique subnetworks were linked together by crucial connector taxa belonging to *Bacillus*, *Mycobacterium*, *Streptomyces* and *Arthrobacter*. This contrasting pattern may indicate that the global microbe-microbe interactions within the complex bacterial community are similar between soybean genotypes, but the key microbe-microbe interactions are genotype-specific.

2.4.4 Consistent rhizosphere effects on bacterial metabolic capacities between soil types and genotype

Functional pathway analysis revealed distinct microbial metabolic capacities in the soybean rhizosphere, and these rhizosphere effects were consistent between different soil types and soybean genotypes. Specifically, bacterial functional pathways related to plant-microbe interactions, biodegradation of xenobiotics, as well as nutrient transformation and transport were significantly enriched in the soybean rhizosphere, while antibiotic biosynthesis, DNA repair and recombination related pathways were reduced. Many of the enriched pathways in the rhizosphere have previously been reported to be essential for the various plant growth promoting functions across several studies (Matilla et al. 2007; Levy et al. 2018; Bulgarelli et al. 2015). For example, flagellar assembly, siderophores and bacterial secretion system were revealed to be involved in induced systemic resistance (Shameer and Prasad 2018). Despite the clear influence of the soil type and soybean genotype on bacterial community composition and microbe-microbe interactions in the soybean rhizosphere, our study identified much overlap in the metabolic capacities of the bacterial communities. This convergence may be due to the functional redundancy of various taxa in the bacterial community (Allison and Martiny 2008). However, this study is limited to inferring functional annotation based on taxonomic classification, and further confirmation of actual rhizosphere microbiome functions is warranted.

Plants are not able to escape from unfavorable conditions, such as being attacked by herbivores or pathogens, due to their sessile nature. During their evolution, plants have developed various strategies to directly or indirectly respond to external stressors by exuding various defense compounds into the rhizosphere for instance (Baetz and Martinoia 2014). To adapt to this specialized habitat, the rhizosphere microbiome may have evolved with increased detoxification activity as reflected by the enhanced degradation pathway of limonene, pinene and naphthalene in our results. This finding is consistent with a former report about the intensive expression of genes involved in oxidative stress response and detoxification in the corn rhizosphere (Matilla et al. 2007). Our functional characterization of the soybean rhizosphere also showed that common carbon metabolism pathways including starch, sucrose, fructose and mannose metabolism were downregulated. As Boris and Jörg stated that most bacteria are characterized with flexible and dynamic carbon-utilization strategy in response to available carbon sources (Görke and Stülke 2008). This decrease in common carbon metabolism pathway could reflect the adaption of the rhizospehree microbiome to the abundant specialized nutrients being supplied by root exudates. This is consistent with the reports of the carbon utilization capacities of several plant growth promoting bacteria. For example, α-pinene can be used by *Pseudomonas fluorescens* as its sole carbon and energy source. Similarly, naphthalene can be utilized as the sole carbon and energy source by several bacterial genera including *Burkholderia*, *Mycobacterium*, *Streptomyces*, *Sphingomonas*, *Pseudomonas*, *Ralstonia*, etc (Seo, Keum, and Li 2009; Wenke, Kai, and Piechulla 2010). Surprisingly, we found that the antibiotic activity were reduced in the rhizosphere, which contradicts previous reports that antibiotic activity of PGPR in rhizosphere are particularly important especially when plants were infected by pathogens (Mahoney, Yin, and Hulbert 2017; Lucas William Mendes et al. 2018; Shameer and Prasad 2018). This difference could be due to the different soil nutrient conditions or lack of pathogen stress in our experiment.

2.5 Conclusions and perspectives

In this study, we provide a detailed characterization of soybean rhizosphere microbiome composition and functional capacity across a number of soybean genotypes and a wild accession. The rhizosphere microbiome composition and microbe-microbe interactions between soybean genotypes and soil types advances our understanding of the modulating role of both factors in the soybean rhizosphere microbiome assembly. This base knowledge primes further studies to use candidate bacteria consortia for synthetic community-based *in vitro* testing of this assembly process and the functional roles of the bacteria. Our results emphasize the importance of comprehensive consideration of native microbe pool, local soil environment and plant genotypes for future microbiome study. Additionally, the significant genotype tuning role in the soybean rhizosphere microbiome assembly indicates that agricultural breeding programs will need to consider integrating host traits participating in beneficial microbiota assembly.

Reference

Agler, Matthew T., Jonas Ruhe, Samuel Kroll, Constanze Morhenn, Sang-Tae Kim, Detlef Weigel, and Eric M. Kemen. 2016. “Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation.” PLoS Biology 14 (1): e1002352.

Allison, Steven D., and Jennifer B. H. Martiny. 2008. “Resistance, Resilience, and Redundancy in Microbial Communities.” Proceedings of the National Academy of Sciences of the United States of America 105 (Supplement 1): 11512–19.

Anderson, Marti J., and Trevor J. Willis. 2003. “Canonical Analysis of Principal Coordinates: A Useful Method of Constrained Ordination for Ecology.” Ecology 84 (2): 511–25.

Asnicar, Francesco, George Weingart, Timothy L. Tickle, Curtis Huttenhower, and Nicola Segata. 2015. “Compact Graphical Representation of Phylogenetic Data and Metadata with GraPhlAn.” PeerJ 3 (June): e1029.

Aßhauer, Kathrin P., Bernd Wemheuer, Rolf Daniel, and Peter Meinicke. 2015. “Tax4Fun: Predicting Functional Profiles from Metagenomic 16S rRNA Data.” Bioinformatics 31 (17): 2882–84.

Baetz, Ulrike, and Enrico Martinoia. 2014. “Root Exudates: The Hidden Part of Plant Defense.” Trends in Plant Science 19 (2): 90–98.

Bakker, Peter A. H. M., Corné M. J. Pieterse, Ronnie de Jonge, and Roeland L. Berendsen. 2018. “The Soil-Borne Legacy.” Cell 172 (6): 1178–80.

Beckers, Bram, Michiel Op De Beeck, Nele Weyens, Wout Boerjan, and Jaco Vangronsveld. 2017. “Structural Variability and Niche Differentiation in the Rhizosphere and Endosphere Bacterial Microbiome of Field-Grown Poplar Trees.” Microbiome 5 (1): 25.

Berendsen, Roeland L., Corné M. J. Pieterse, and Peter A. H. M. Bakker. 2012. “The Rhizosphere Microbiome and Plant Health.” Trends in Plant Science 17 (8): 478–86.

Bulgarelli, Davide, Ruben Garrido-Oter, Philipp C. Münch, Aaron Weiman, Johannes Dröge, Yao Pan, Alice C. McHardy, and Paul Schulze-Lefert. 2015. “Structure and Function of the Bacterial Root Microbiota in Wild and Domesticated Barley.” Cell Host & Microbe 17 (3): 392–403.

Bulgarelli, Davide, Klaus Schlaeppi, Stijn Spaepen, Emiel Ver Loren van Themaat, and Paul Schulze-Lefert. 2013. “Structure and Functions of the Bacterial Microbiota of Plants.” Annual Review of Plant Biology 64 (January): 807–38.

Cardinale, Massimiliano, Martin Grube, Armin Erlacher, Julian Quehenberger, and Gabriele Berg. 2015. “Bacterial Networks and Co-Occurrence Relationships in the Lettuce Root Microbiota.” Environmental Microbiology 17 (1): 239–52.

Castrillo, Gabriel, Paulo José Pereira Lima Teixeira, Sur Herrera Paredes, Theresa F. Law, Laura de Lorenzo, Meghan E. Feltcher, Omri M. Finkel, et al. 2017. “Root Microbiota Drive Direct Integration of Phosphate Stress and Immunity.” Nature 543 (7646): 513–18.

Clarke, K. R. 1993. “Non-parametric Multivariate Analyses of Changes in Community Structure.” Austral Ecology 18 (1): 117–43.

Csardi, Gabor, and Tamas Nepusz. 2006. “The Igraph Software Package for Complex Network Research.” InterJournal, Complex Systems 1695 (5): 1–9.

Dutta, Swarnalee, and Appa Rao Podile. 2010. “Plant Growth Promoting Rhizobacteria (PGPR): The Bugs to Debug the Root Zone.” Critical Reviews in Microbiology 36 (3): 232–44.

Edgar, Robert C., Brian J. Haas, Jose C. Clemente, Christopher Quince, and Rob Knight. 2011. “UCHIME Improves Sensitivity and Speed of Chimera Detection.” Bioinformatics 27 (16): 2194–2200.

Edwards, Joseph, Cameron Johnson, Christian Santos-Medellín, Eugene Lurie, Natraj Kumar Podishetty, Srijak Bhatnagar, Jonathan A. Eisen, and Venkatesan Sundaresan. 2015. “Structure, Variation, and Assembly of the Root-Associated Microbiomes of Rice.” Proceedings of the National Academy of Sciences of the United States of America 112 (8): E911–20.

Fitzpatrick, Connor R., Julia Copeland, Pauline W. Wang, David S. Guttman, Peter M. Kotanen, and Marc T. J. Johnson. 2018. “Assembly and Ecological Function of the Root Microbiome across Angiosperm Plant Species.” Proceedings of the National Academy of Sciences of the United States of America 115 (6): E1157–65.

Friedman, Jonathan, and Eric J. Alm. 2012. “Inferring Correlation Networks from

Genomic Survey Data.” PLoS Computational Biology 8 (9): e1002687.

Goh, Chooi-Hua, Debora F. Veliz Vallejos, Adrienne B. Nicotra, and Ulrike Mathesius. 2013. “The Impact of Beneficial Plant-Associated Microbes on Plant Phenotypic Plasticity.” Journal of Chemical Ecology 39 (7): 826–39.

Görke, Boris, and Jörg Stülke. 2008. “Carbon Catabolite Repression in Bacteria: Many Ways to Make the Most out of Nutrients.” Nature Reviews. Microbiology 6 (8): 613–24.

Gupta, Govind, Shailendra Singh Parihar, Narendra Kumar Ahirwar, Sunil Kumar Snehi, and Vinod Singh. 2015. “Plant Growth Promoting Rhizobacteria (PGPR): Current and Future Prospects for Development of Sustainable Agriculture.” J Microb Biochem Technol 7 (2): 096–102.

Haney, Cara H., Buck S. Samuel, Jenifer Bush, and Frederick M. Ausubel. 2015. “Associations with Rhizosphere Bacteria Can Confer an Adaptive Advantage to Plants.” Nature Plants 1 (6): 15051.

Hartman, Glen L., Ellen D. West, and Theresa K. Herman. 2011. “Crops That Feed the World 2. Soybean-Worldwide Production, Use, and Constraints Caused by Pathogens and Pests.” Food Security 3 (1): 5–17.

Hartman, Kyle, Marcel Ga van der Heijden, Valexia Roussely-Provent, Jean-Claude Walser, and Klaus Schlaeppi. 2017. “Deciphering Composition and Function of the Root Microbiome of a Legume Plant.” Microbiome 5 (1): 2.

Hassani, M. Amine, Paloma Durán, and Stéphane Hacquard. 2018. “Microbial Interactions within the Plant Holobiont.” Microbiome 6 (1): 58.

Hayashi, Masaki, Yuichi Saeki, Michiyo Haga, Kyuya Harada, Hiroshi Kouchi, and Yosuke Umehara. 2012. “Rj (rj) Genes Involved in Nitrogen-Fixing Root Nodule Formation in Soybean.” Breeding Science 61 (5): 544–53.

Herridge, David F., Mark B. Peoples, and Robert M. Boddey. 2008. “Global Inputs of Biological Nitrogen Fixation in Agricultural Systems.” Plant and Soil 311 (1): 1–18.

Hill, Jason, Erik Nelson, David Tilman, Stephen Polasky, and Douglas Tiffany. 2006. “Environmental, Economic, and Energetic Costs and Benefits of Biodiesel and Ethanol Biofuels.” Proceedings of the National Academy of Sciences of the United States of America 103 (30): 11206–10.

Hunt, S., and D. B. Layzell. 1993. “Gas Exchange of Legume Nodules and the Regulation of Nitrogenase Activity.” Annual Review of Plant Physiology and Plant Molecular Biology 44 (1): 483–511.

Kolde, Raivo. 2015. “Pheatmap: Pretty Heatmaps. R Package Version 1.0. 8.”

Kozich, James J., Sarah L. Westcott, Nielson T. Baxter, Sarah K. Highlander, and Patrick D. Schloss. 2013. “Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform.” Applied and Environmental Microbiology 79 (17): 5112–20.

Lebeis, Sarah L., Sur Herrera Paredes, Derek S. Lundberg, Natalie Breakfield, Jase

Gehring, Meredith McDonald, Stephanie Malfatti, et al. 2015. “Salicylic Acid Modulates Colonization of the Root Microbiome by Specific Bacterial Taxa.” Science 349 (6250): 860–64.

Levy, Asaf, Isai Salas Gonzalez, Maximilian Mittelviefhaus, Scott Clingenpeel, Sur Herrera Paredes, Jiamin Miao, Kunru Wang, et al. 2018. “Genomic Features of Bacterial Adaptation to Plants.” Nature Genetics 50 (1): 138–50.

Love, Michael I., Wolfgang Huber, and Simon Anders. 2014. “Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2.” Genome Biology 15 (12): 550.

Lundberg, Derek S., Sarah L. Lebeis, Sur Herrera Paredes, Scott Yourstone, Jase Gehring, Stephanie Malfatti, Julien Tremblay, et al. 2012. “Defining the Core Arabidopsis Thaliana Root Microbiome.” Nature 488 (7409): 86–90.

Lundberg, Derek S., Scott Yourstone, Piotr Mieczkowski, Corbin D. Jones, and Jeffery L. Dangl. 2013. “Practical Innovations for High-Throughput Amplicon Sequencing.” Nature Methods 10 (10): 999–1002.

Lu, Tao, Mingjing Ke, W. J. G. M. Peijnenburg, Youchao Zhu, Meng Zhang, Liwei Sun, Zhengwei Fu, and Haifeng Qian. 2018. “Investigation of Rhizospheric Microbial Communities in Wheat, Barley, and Two Rice Varieties at the Seedling Stage.” Journal of Agricultural and Food Chemistry 66 (11): 2645–53.

Mahoney, Aaron K., Chuntao Yin, and Scot H. Hulbert. 2017. “Community Structure, Species Variation, and Potential Functions of Rhizosphere-Associated Bacteria of Different Winter Wheat (*Triticum Aestivum*) Cultivars.” Frontiers in Plant Science 8 (February): 132.

Matilla, Miguel A., Manuel Espinosa-Urgel, José J. Rodríguez-Herva, Juan L. Ramos, and María Isabel Ramos-González. 2007. “Genomic Analysis Reveals the Major Driving Forces of Bacterial Life in the Rhizosphere.” Genome Biology 8 (9): R179.

Mendes, Lucas William, Jos M. Raaijmakers, Mattias de Hollander, Rodrigo Mendes, and Siu Mui Tsai. 2018. “Influence of Resistance Breeding in Common Bean on Rhizosphere Microbiome Composition and Function.” The ISME Journal 12 (1): 212–24.

Mendes, Lucas W., Eiko E. Kuramae, Acácio A. Navarrete, Johannes A. van Veen, and Siu M. Tsai. 2014. “Taxonomical and Functional Microbial Community Selection in Soybean Rhizosphere.” The ISME Journal 8 (8): 1577–87.

“Monthly Biodiesel Production Report - Energy Information Administration.” n.d. Accessed October 8, 2018. https://www.eia.gov/biofuels/biodiesel/production/.

Neumann, Günter, and Enrico Martinoia. 2002. “Cluster Roots-an Underground Adaptation for Survival in Extreme Environments.” Trends in Plant Science 7 (4): 162–67.

Panke-Buisse, Kevin, Angela C. Poole, Julia K. Goodrich, Ruth E. Ley, and Jenny Kao-Kniffin. 2015. “Selection on Soil Microbiomes Reveals Reproducible Impacts on

Plant Function.” The ISME Journal 9 (4): 980–89.

Peiffer, Jason A., Aymé Spor, Omry Koren, Zhao Jin, Susannah Green Tringe, Jeffery L. Dangl, Edward S. Buckler, and Ruth E. Ley. 2013. “Diversity and Heritability of the Maize Rhizosphere Microbiome under Field Conditions.” Proceedings of the National Academy of Sciences of the United States of America 110 (16): 6548–53.

Pérez-Jaramillo, Juan E., Víctor J. Carrión, Mirte Bosse, Luiz F. V. Ferrão, Mattias de Hollander, Antonio A. F. Garcia, Camilo A. Ramírez, Rodrigo Mendes, and Jos M. Raaijmakers. 2017. “Linking Rhizosphere Microbiome Composition of Wild and Domesticated Phaseolus Vulgaris to Genotypic and Root Phenotypic Traits.” The ISME Journal 11 (10): 2244–57.

Pérez-Jaramillo, Juan E., Rodrigo Mendes, and Jos M. Raaijmakers. 2016. “Impact of Plant Domestication on Rhizosphere Microbiome Assembly and Functions.” Plant Molecular Biology 90 (6): 635–44.

Philippot, Laurent, Jos M. Raaijmakers, Philippe Lemanceau, and Wim H. van der Putten. 2013. “Going back to the Roots: The Microbial Ecology of the Rhizosphere.” Nature Reviews. Microbiology 11 (11): 789–99.

Pieterse, Corné M. J., Ronnie de Jonge, and Roeland L. Berendsen. 2016. “The Soil-Borne Supremacy.” Trends in Plant Science 21 (3): 171–73.

Poudel, R., A. Jumpponen, D. C. Schlatter, T. C. Paulitz, B. B. Mcspadden Gardener, L. L. Kinkel, and K. A. Garrett. 2016. “Microbiome Networks: A Systems Framework for Identifying Candidate Microbial Assemblages for Disease Management." Phytopathology 106 (10): 1083–96.

Rognes, Torbjørn, Tomáš Flouri, Ben Nichols, Christopher Quince, and Frédéric Mahé. 2016. "VSEARCH: A Versatile Open Source Tool for Metagenomics." PeerJ 4 (October): e2584.

Salvagiotti, F., K. G. Cassman, J. E. Specht, D. T. Walters, A. Weiss, and A. Dobermann. 2008. "Nitrogen Uptake, Fixation and Response to Fertilizer N in Soybeans: A Review." Field Crops Research 108 (1): 1–13.

Samad, Abdul, Friederike Trognitz, Stéphane Compant, Livio Antonielli, and Angela Sessitsch. 2017. "Shared and Host-Specific Microbiome Diversity and Functioning of Grapevine and Accompanying Weed Plants." Environmental Microbiology 19 (4): 1407–24.

Sasse, Joelle, Enrico Martinoia, and Trent Northen. 2018. "Feed Your Friends: Do Plant Exudates Shape the Root Microbiome?" Trends in Plant Science 23 (1): 25–41.

Schlaeppi, Klaus, and Davide Bulgarelli. 2015. "The Plant Microbiome at Work." Molecular Plant-Microbe Interactions: MPMI 28 (3): 212–17.

Segata, Nicola, Jacques Izard, Levi Waldron, Dirk Gevers, Larisa Miropolsky, Wendy S. Garrett, and Curtis Huttenhower. 2011. "Metagenomic Biomarker Discovery and Explanation." Genome Biology 12 (6): R60.

Seo, Jong-Su, Young-Soo Keum, and Qing X. Li. 2009. "Bacterial Degradation of

Aromatic Compounds." International Journal of Environmental Research and Public Health 6 (1): 278–309.

Shameer, Syed, and T. N. V. K. V. Prasad. 2018. "Plant Growth Promoting Rhizobacteria for Sustainable Agricultural Practices with Special Reference to Biotic and Abiotic Stresses." Plant Growth Regulation 84 (3): 603–15.

Souza, Rafael Soares Correa de, Vagner Katsumi Okura, Jaderson Silveira Leite Armanhi, Beatriz Jorrín, Núria Lozano, Márcio José da Silva, Manuel González-Guerrero, et al. 2016. "Unlocking the Bacterial and Fungal Communities Assemblages of Sugarcane Microbiome." Scientific Reports 6 (June): 28774.

Sugiyama, Akifumi, Yoshikatsu Ueda, Takahiro Zushi, Hisabumi Takase, and Kazufumi Yazaki. 2014. "Changes in the Bacterial Community of Soybean Rhizospheres during Growth in the Field." PloS One 9 (6): e100709.

Takahashi, Shunsuke, Junko Tomita, Kaori Nishioka, Takayoshi Hisada, and Miyuki Nishijima. 2014. "Development of a Prokaryotic Universal Primer for Simultaneous Analysis of Bacteria and Archaea Using next-Generation Sequencing." PloS One 9 (8): e105592.

Timm, C. M., K. R. Carter, A. A. Carrell, S. R. Jun, and S. S. Jawdy. 2018. "Abiotic Stresses Shift Belowground Populus-Associated Bacteria toward a Core Stress Microbiome." MSystem 3(1): e00070–17.

Turner, Thomas R., Karunakaran Ramakrishnan, John Walshaw, Darren Heavens, Mark Alston, David Swarbreck, Anne Osbourn, Alastair Grant, and Philip S. Poole. 2013. “Comparative Metatranscriptomics Reveals Kingdom Level Changes in the Rhizosphere Microbiome of Plants.” The ISME Journal 7 (12): 2248–58.

Vance, C. P. 2001. “Symbiotic Nitrogen Fixation and Phosphorus Acquisition. Plant Nutrition in a World of Declining Renewable Resources.” Plant Physiology 127 (2): 390–97.

Warembourg, F. R., C. Roumet, and F. Lafont. 2003. “Differences in Rhizosphere Carbon-Partitioning among Plant Species of Different Families.” Plant and Soil 256 (2): 347–57.

Weisskopf, Laure, Stefanie Heller, and Leo Eberl. 2011. “Burkholderia Species Are Major Inhabitants of White Lupin Cluster Roots.” Applied and Environmental Microbiology 77 (21): 7715–20.

Wenke, Katrin, Marco Kai, and Birgit Piechulla. 2010. “Belowground Volatiles Facilitate Interactions between Plant Roots and Soil Organisms.” Planta 231 (3): 499–506.

White, Laura J., Karuppaiyan Jothibasu, R. Neil Reese, Volker S. Brözel, and Senthil Subramanian. 2015. “Spatio Temporal Influence of Isoflavonoids on Bacterial Diversity in the Soybean Rhizosphere.” Molecular Plant-Microbe Interactions: MPMI 28 (1): 22–29.

Xiao, Xiao, Weimin Chen, Le Zong, Jun Yang, Shuo Jiao, Yanbing Lin, Entao Wang, and Gehong Wei. 2017. “Two Cultivated Legume Plants Reveal the Enrichment

Process of the Microbiome in the Rhizocompartments.” Molecular Ecology 26 (6): 1641–51.

Zachow, Christin, Henry Müller, Ralf Tilcher, and Gabriele Berg. 2014. “Differences between the Rhizosphere Microbiome of Beta Vulgaris Ssp. Maritima - Ancestor of All Beet Crops - and Modern Sugar Beets.” Frontiers in Microbiology 5: 415.

Zahran, H. H. 1999. “Rhizobium-Legume Symbiosis and Nitrogen Fixation under Severe Conditions and in an Arid Climate.” Microbiology and Molecular Biology Reviews: MMBR 63 (4): 968–89.

Zgadzaj, Rafal, Ruben Garrido-Oter, Dorthe Bodker Jensen, Anna Koprivova, Paul Schulze-Lefert, and Simona Radutoiu. 2016. “Root Nodule Symbiosis in Lotus Japonicus Drives the Establishment of Distinctive Rhizosphere, Root, and Nodule Bacterial Communities.” Proceedings of the National Academy of Sciences of the United States of America 113 (49): E7996–8005.

Zhalnina, Kateryna, Katherine B. Louie, Zhao Hao, Nasim Mansoori, Ulisses Nunes da Rocha, Shengjing Shi, Heejung Cho, et al. 2018. “Dynamic Root Exudate Chemistry and Microbial Substrate Preferences Drive Patterns in Rhizosphere Microbial Community Assembly.” Nature Microbiology 3 (4): 470–80.

Zhao, Jing, Jiabing Fu, Hong Liao, Yong He, Hai Nian, Yueming Hu, Linjuan Qiu, Yinsan Dong, and Xiaolong Yan. 2004. “Characterization of Root Architecture in an Applied Core Collection for Phosphorus Efficiency of Soybean Germplasm.” Chinese Science Bulletin 49 (15): 1611–20.

Appendices 2

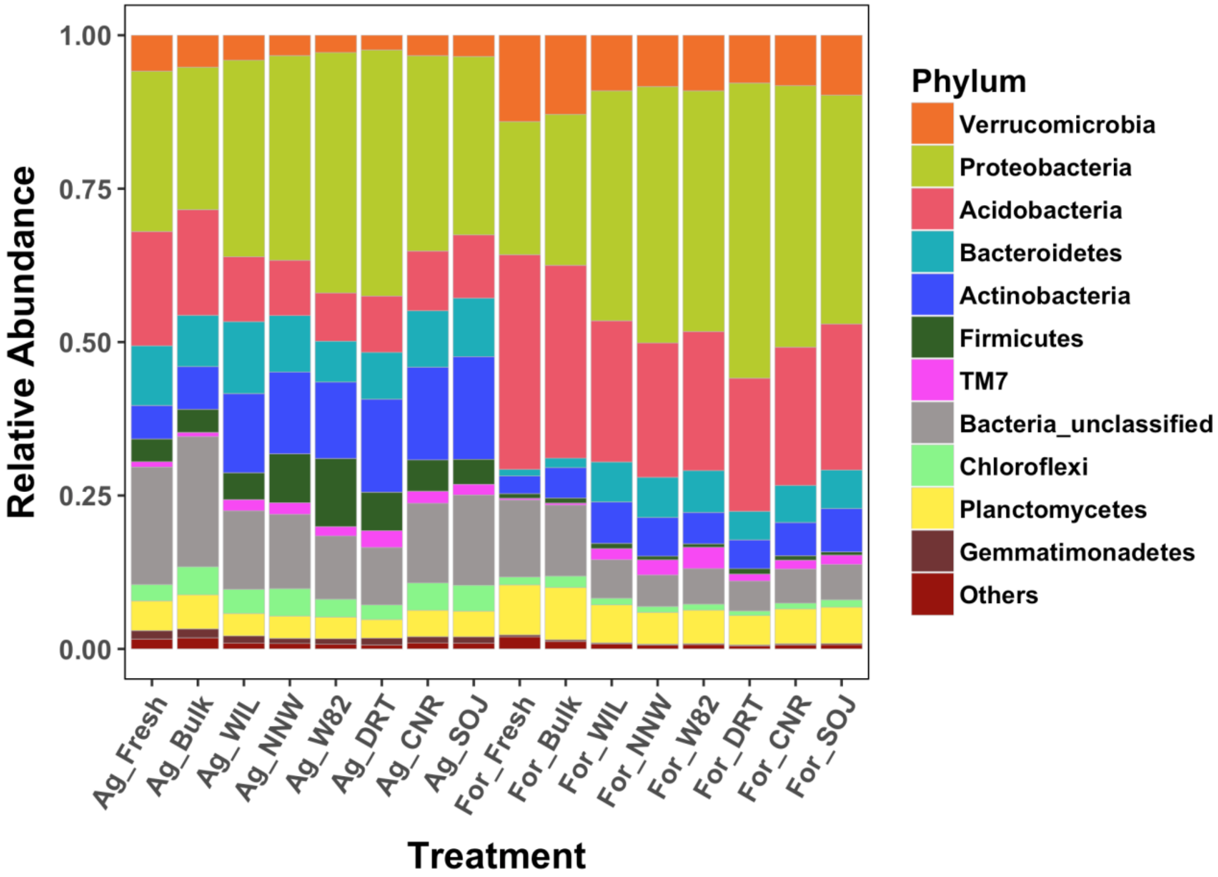


Fig. 2.1 Bacterial community composition at phylum level.

Bacterial phyla with relative abundance smaller than 1% across 20% of samples were grouped together to form the “Others” category. Fresh soil was flash frozen immediately after field collection, while bulk soil was treated the same as and collected parallel to rhizosphere samples.

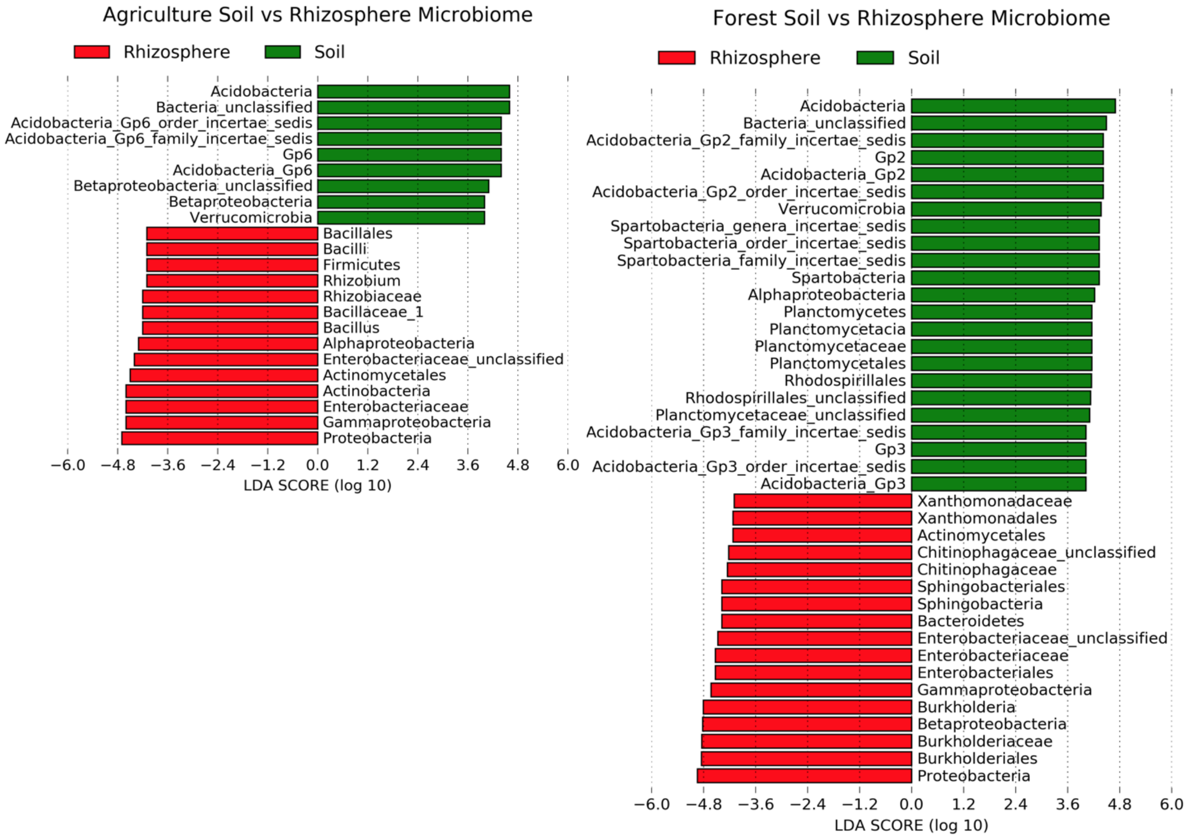
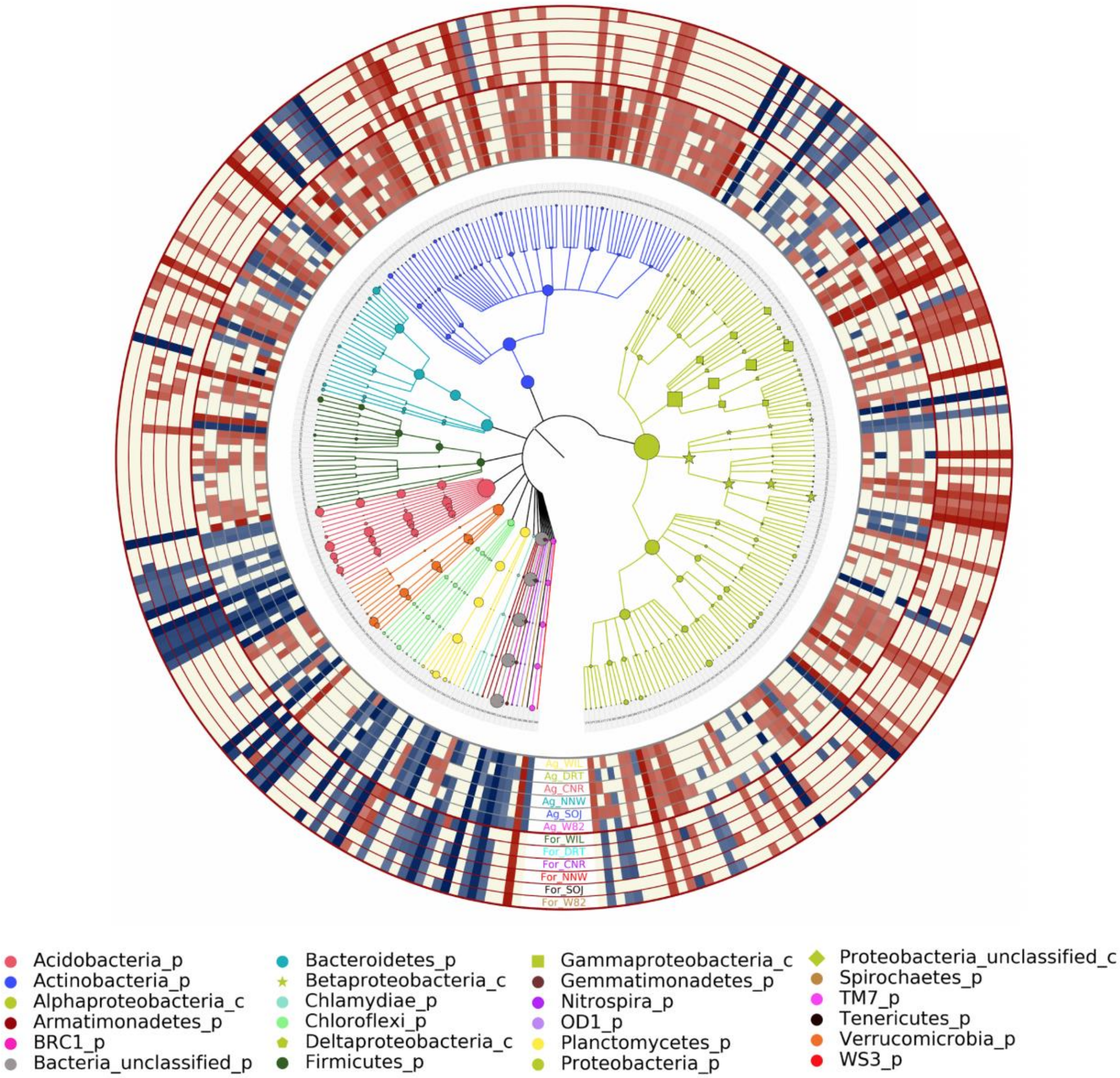


Fig. 2.2 Differential abundance between soil and rhizosphere by LefSe analysis at LDA score >4.

In this LefSe analysis, soil samples (including both fresh and bulk samples) were treated as controls. A negative LDA score represents depletion in soil and enrichment in rhizosphere (red) and a positive LDA score represents the opposite (green).

Fig. 2.3 The enrichment and depletion of bacteria by genera in the soybean rhizosphere.

The inside dendrogram represents the taxonomic tree of all bacterial genera with significantly different abundance between soil and rhizosphere, with color indicating phylum. *Proteobacteria* (green) were subset to class level, with circle, star, pentagon, square and diamond representing *Alpha*-, *Beta*-, *Delta*-, *Gamma*- and Unclassified- *Proteobacteria* respectively. The number at the end of each branch represents the corresponding bacterial genus as annotated along the list along each side of the plot. A detailed annotation list could be found in Table S2.1. The enrichment/depletion of each genus in the soybean rhizosphere is depicted in the external heatmap ring, with red indicating enrichment, blue representing depletion, and yellow indicating no significant difference. The darker the color of each block, the stronger the corresponding enrichment/depletion, which is scaled based on corresponding LDA score.



Enrichment and Depletion of Bacterial Genera in Rhizosphere

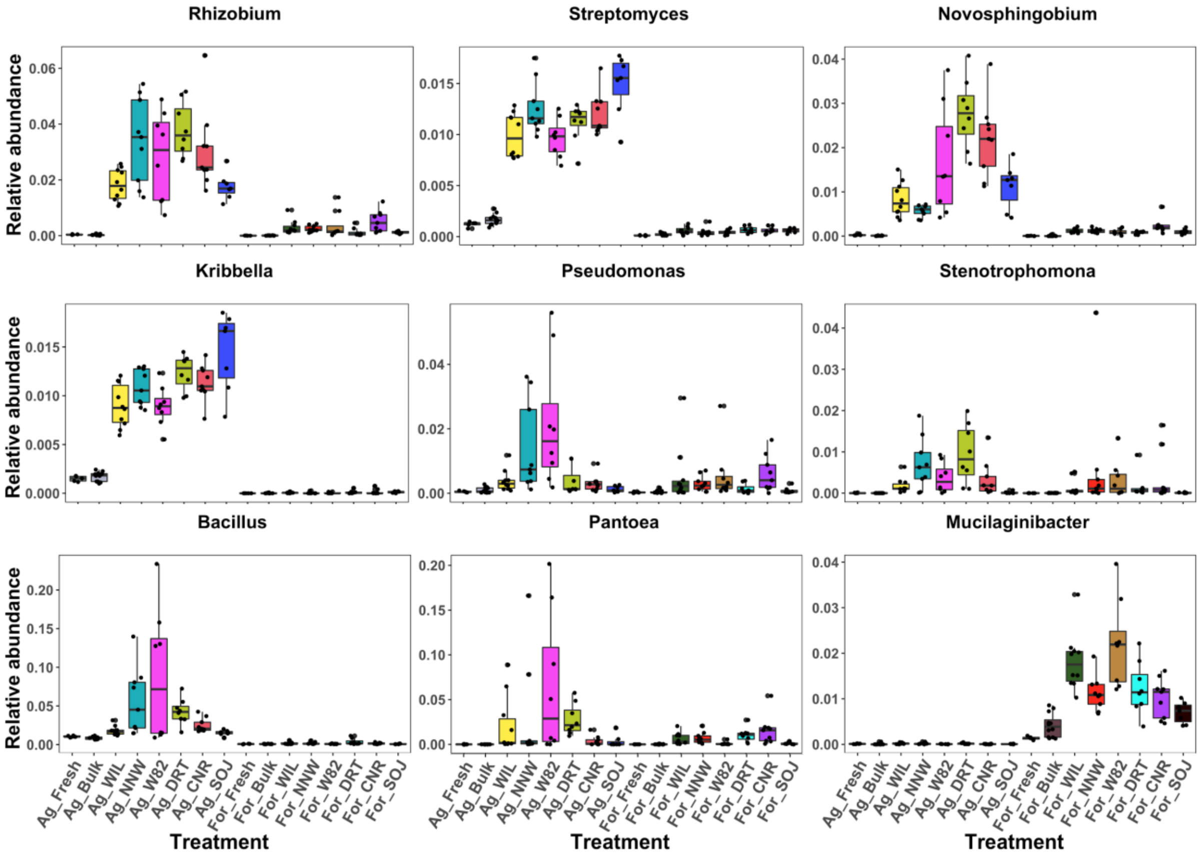


Fig. 2.4 Boxplot of bacterial genus abundance between treatments.

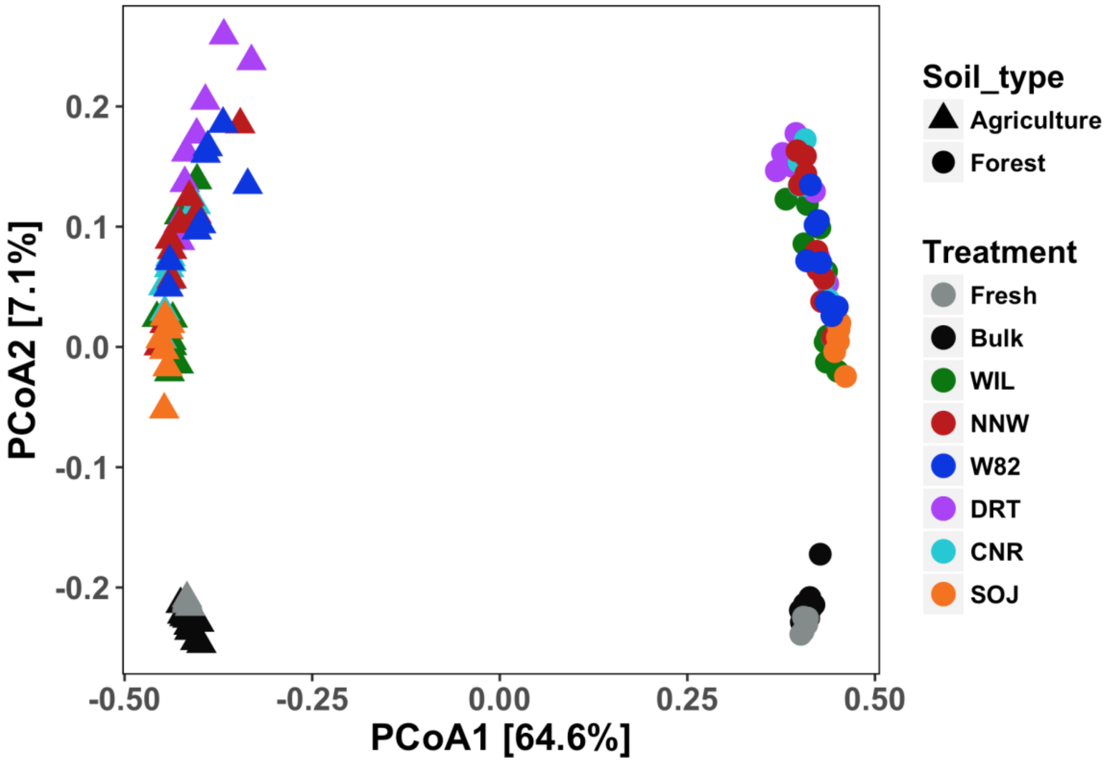


Fig. 2.5 Bacterial community composition between treatment.

Agriculture and forest soil types were represented by triangle and circle correspondingly. Different colors of the points represent different treatments.

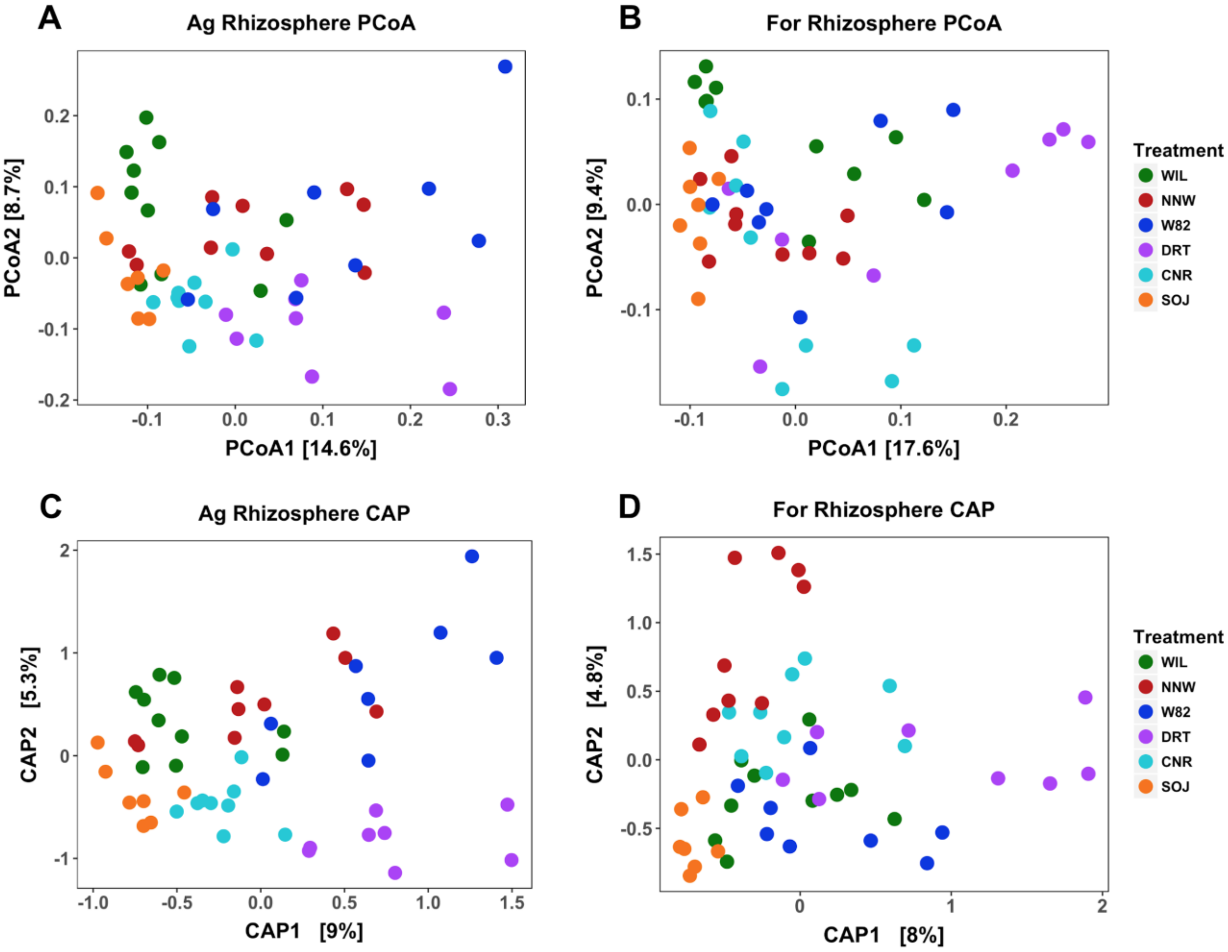


Fig. 2.6 Genotype impacts on soybean rhizosphere microbiome assembly.

Principal coordinate analysis (PCoA) plot of soybean rhizosphere microbial community grown in agriculture soil (A) and forest soil (B) demonstrates little pattern associated with genotype. In contrast, soybean rhizosphere microbial community difference between genotypes as depicted by canonical analysis of principal coordinates (CAP) has more clear genotype-specific patterns, with C and D representing rhizosphere samples grown in agriculture and forest soil, respectively.

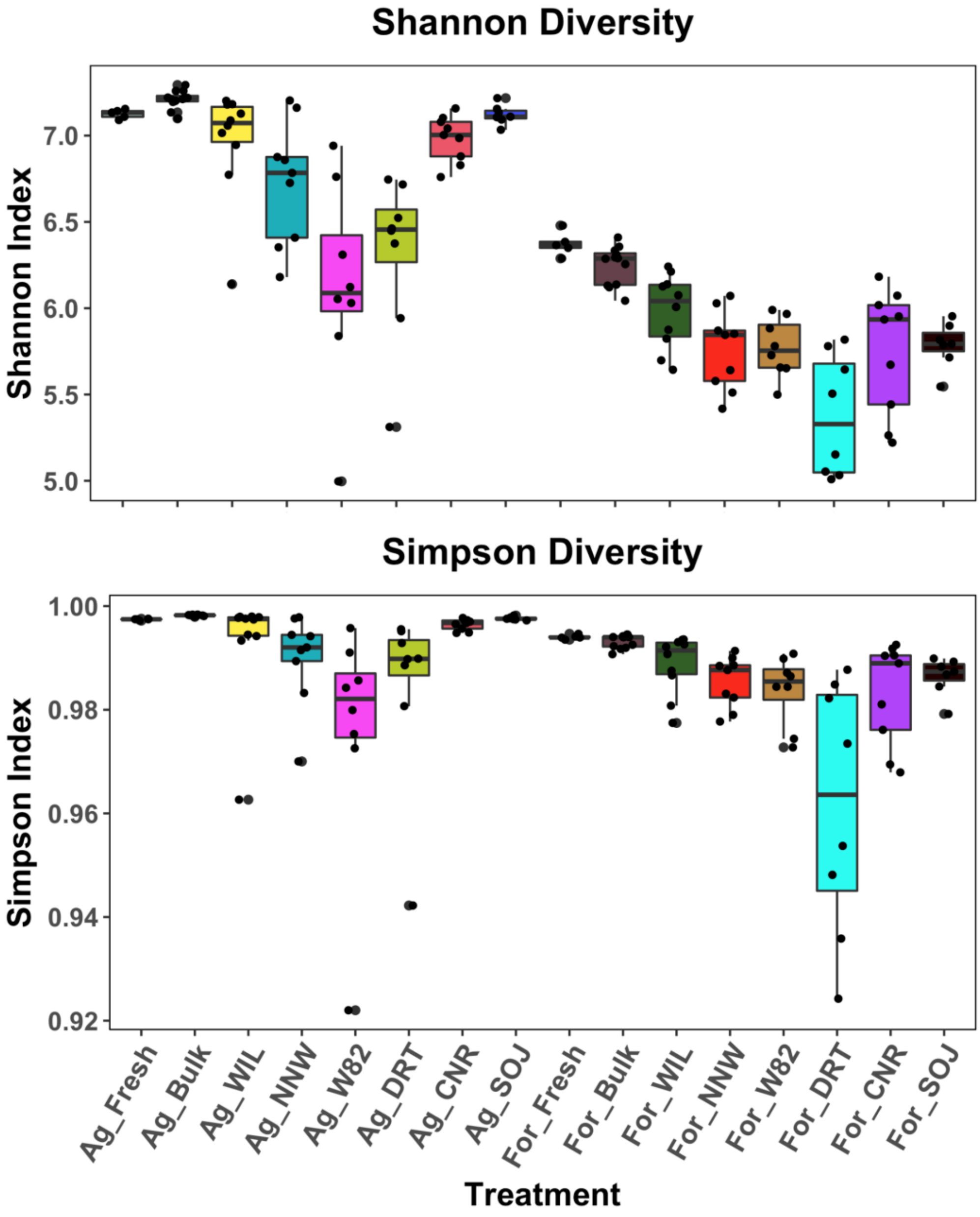


Fig. 2.7 Rhizosphere effects on microbiome diversity.

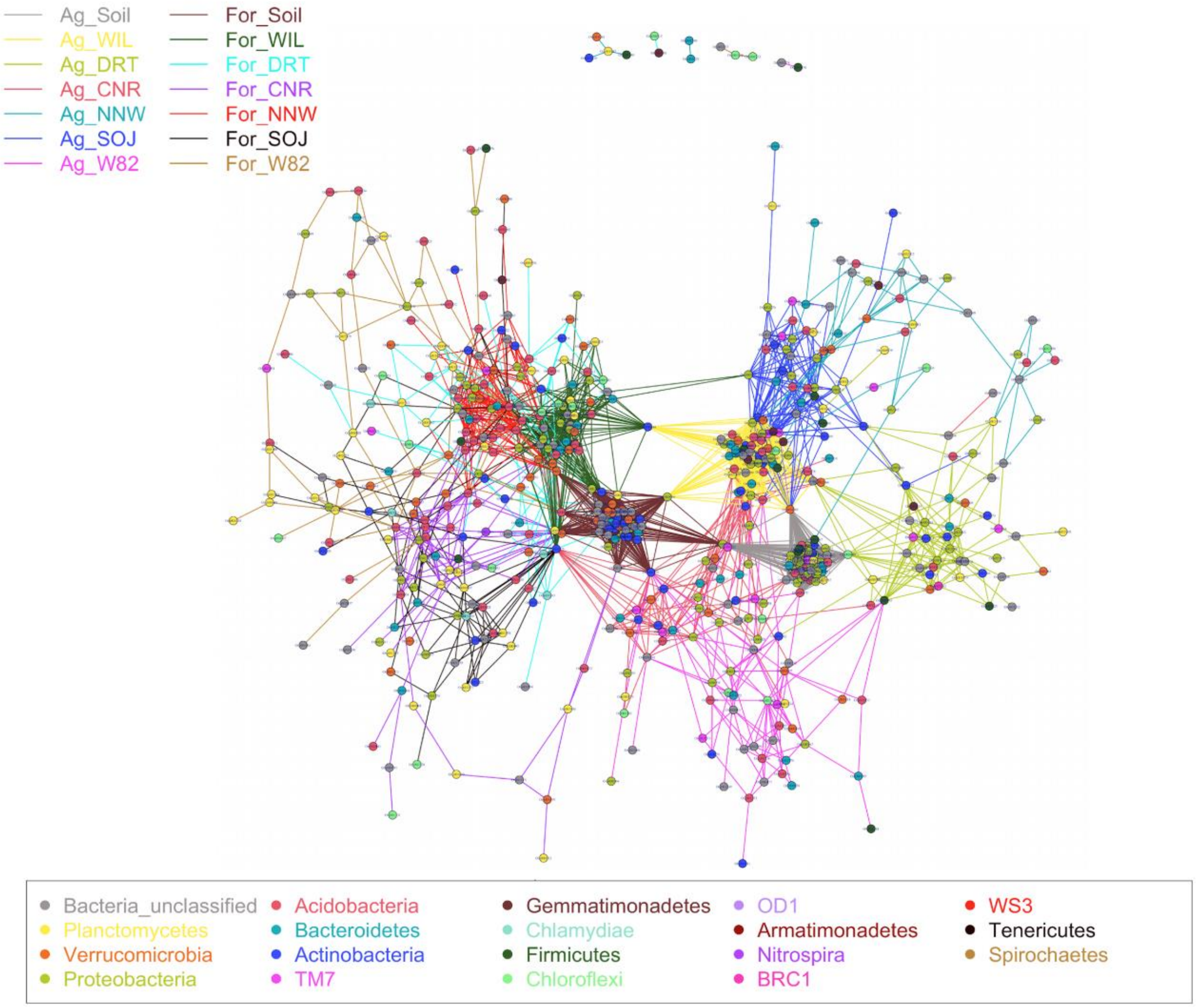


Fig. 2.8 Modulation of microbial networks by soybean genotypes and soil source.

In the above network, OTUs were represented by individual nodes, with colors indicating phylum. Edge color denotes the treatment. When one edge was shared between treatment, a mixed color was used to define that particular edge. OTU numbers are labeled for each node and their corresponding taxonomic information can be found in Table S2.3.

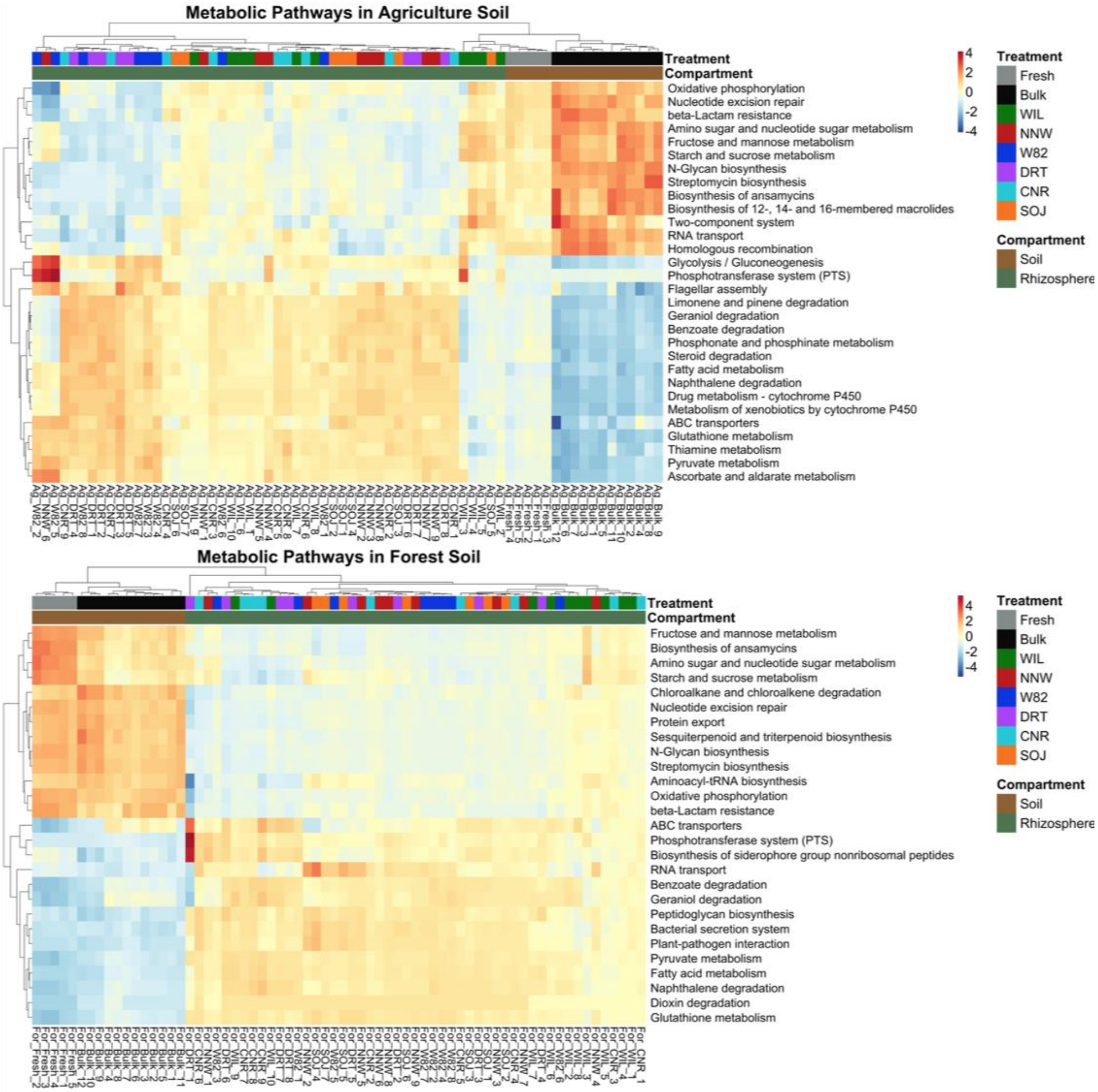


Fig. 2.9 Rhizosphere effects on soybean microbiome metabolic capacity.

Metabolic pathways that differed significantly between soil and rhizosphere were used to generate a heatmap. Both samples and pathways were clustered based on euclidean distance. The abundance of each pathway was scaled to the same range (-4, 4), with red and blue colors representing relatively higher and lower abundance respectively.

Table 2.1 Soybean genotype inventories and specific characters.

genotype Abbr.	Plant Inventory	Maturity Group	Cultivar or distinguishing character
WIL	548631	III	Williams
DRT	416937	VI	Drought-tolerant with different root morphology
CNR	TN09-029	IV	Soybean cyst nematode-resistant
NNW	634765	III	Non-nodulating mutant of Williams
SOJ	407305	V	*Glycine soja* undomesticated progenitor
W82	518671	III	Williams 82

Table 2.2 Network topological characteristics.

Global statistics were calculated based on co-occurrence network comprising all significant microbe-microbe correlations at α<0.001 while top 50 statistics were calculated based on simplified network that including only the top 50 nodes with the most connections to other microbes.

Soil type	**Network type**	**Network topologies**	**Soil**	**WIL**	**DRT**	**CNR**	**NNW**	**SOJ**	**W82**
Agriculture	Global	Positive edges[a] (%)	54.64	56.69	58.74	57.33	57.68	55.91	57.77
		Edge density[b] (%)	2.35	1.58	0.76	0.81	0.64	0.93	0.73
		Ave. degree[c]	25.68	16.83	8.46	8.93	6.97	9.32	6.11
		Betweenness[d]	1092.03	1294.41	1588.35	1576.23	1691.40	1471.82	1405.33
	Top 50	Positive edges[a] (%)	48.45	50.66	93.28	48.75	62.26	66.67	90.91
		Edge density[b] (%)	76.49	49.47	11.88	13.61	6.79	15.54	8.42
		Ave. degree[c]	37.48	24.24	5.58	6.53	2.65	7.15	4.04
		Betweenness[d]	5.76	12.38	34.02	34.73	29.20	34.47	46.34
Forest	Global	Positive edges[a] (%)	56.25	55.68	56.21	55.42	57.60	54.81	55.70
		Edge density[b] (%)	1.49	0.88	0.73	0.68	0.77	0.75	0.74
		Ave. degree[c]	16.77	9.75	8.38	7.43	8.75	8.54	8.59
		Ave. Betweenness[d]	1273.30	1541.86	1653.03	1626.60	1611.71	1589.82	1626.15
	Top 50	Positive edges[a] (%)	58.03	60.66	70.33	94.57	84.72	45.54	77.27
		Edge density[b] (%)	63.02	24.90	7.74	10.69	19.61	9.76	6.67
		Ave. degree[c]	30.88	12.20	3.71	4.38	8.83	4.39	2.93
		Ave. Betweenness[d]	9.06	23.30	55.98	56.24	27.04	47.76	62.22

[a] Positive ratio represents the ratio of positive microbe-microbe correlations out of all interactions within the network.

[b] Edge density was calculated as the ratio of detected edge numbers to the theoretical maximum edge numbers, indicating the connectiveness between nodes.

[c] Ave. degree was defined as the mean connection degree across all nodes within a network.

[d] Ave. Betweenness was defined by the average number of shortest paths going through all vertice within a network.

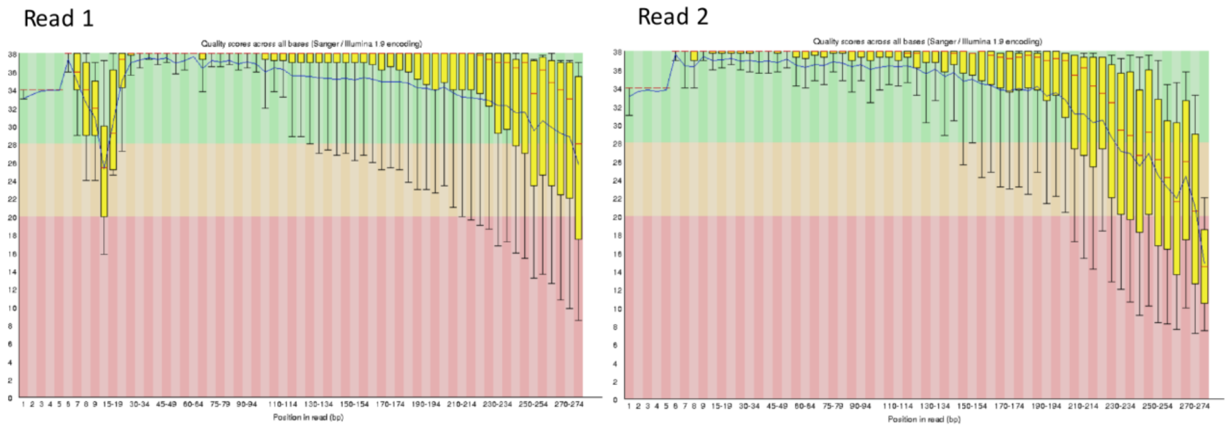


Fig. S2.1 Sequence quality analysis using fastQC.

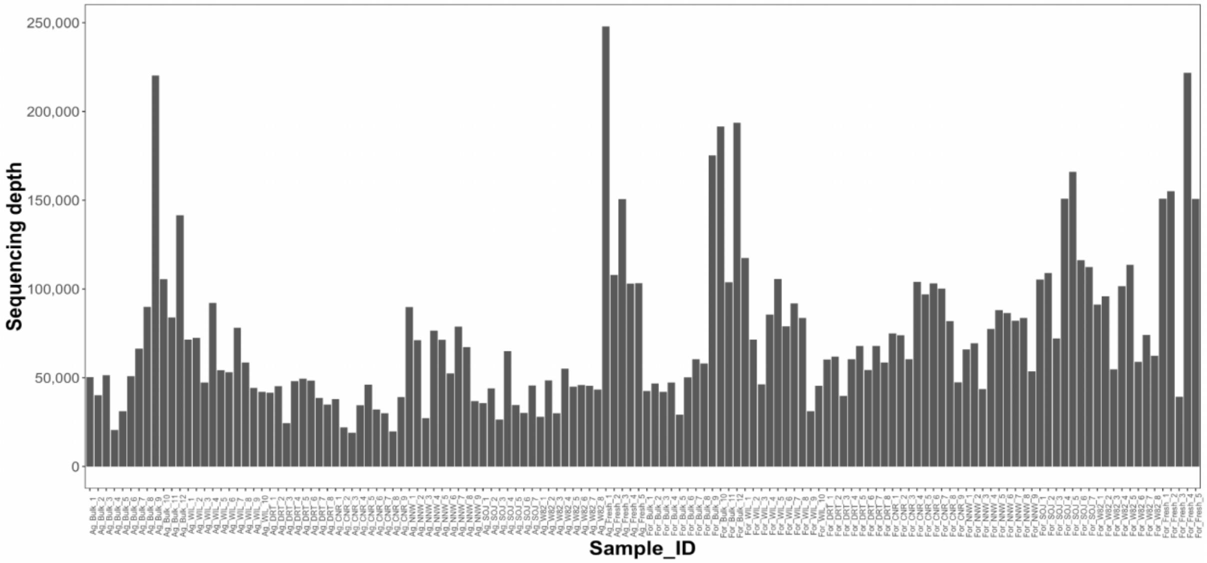


Fig. S2.2 Sequencing depth distribution across all samples.

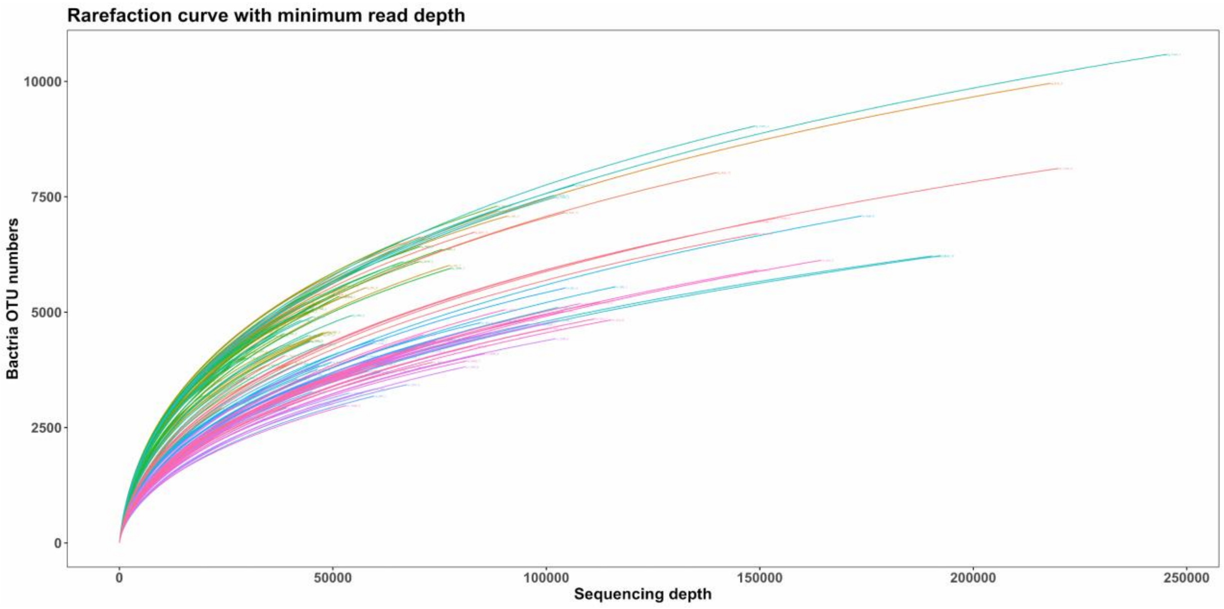


Fig. S2.3 Rarefaction curve across all samples.

The sample ID were labeled at the end of each line.

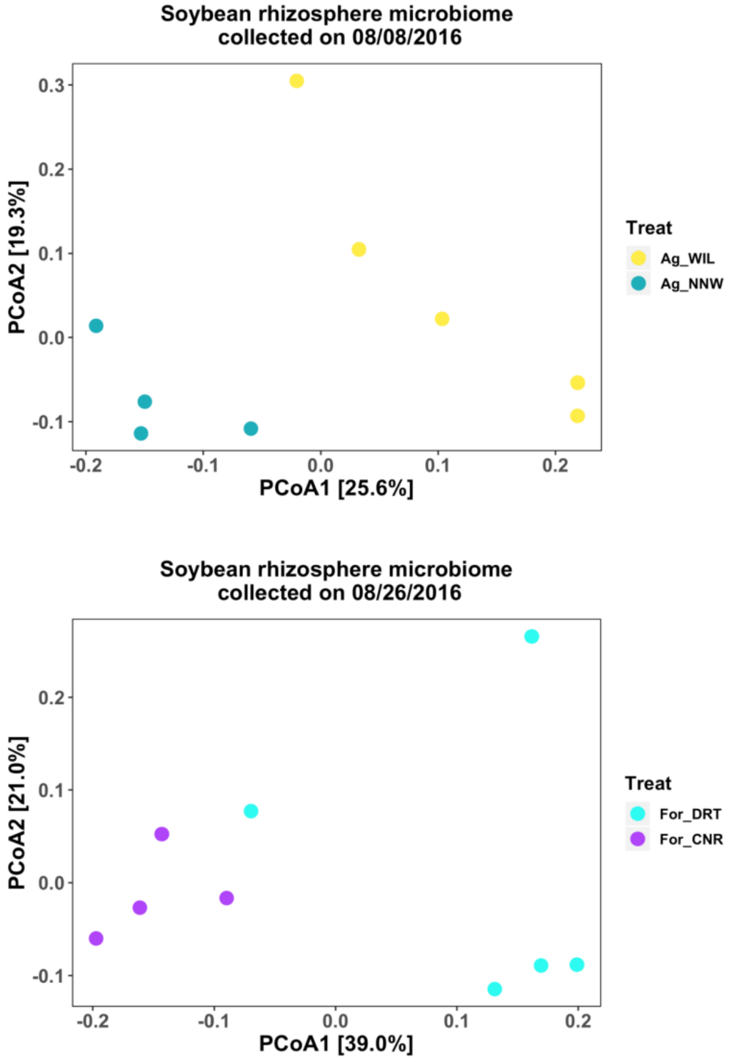


Fig. S2.4 Genotype effects on soybean rhizosphere microbiome by comparing samples collected on the same date.

To provide an example of pure genotype effect, rhizosphere samples belongs to William (WIL) vs non-nodulating William (NNW) mutant growing in agriculture soil that collected on 08-08-2016 and cyst nematode resistant (CNR) vs drought tolerant (DRT) collected on 08-26-2018 were compared and visualized using PCoA plot.

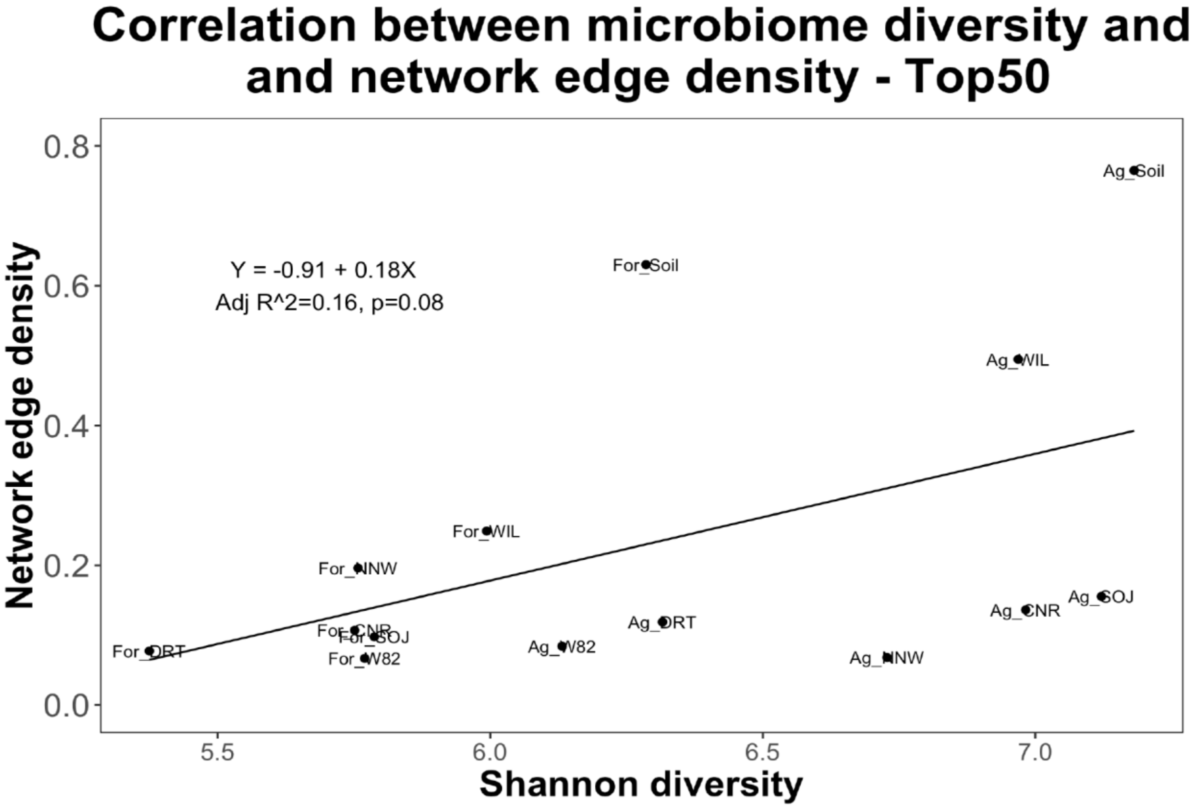


Fig. S2.5 Correlation analysis between microbial Shannon diversities and network edge densities.

The below correlation was calculated based on top50 network in which the first 50 nodes with the highest connections with other microbes were included.

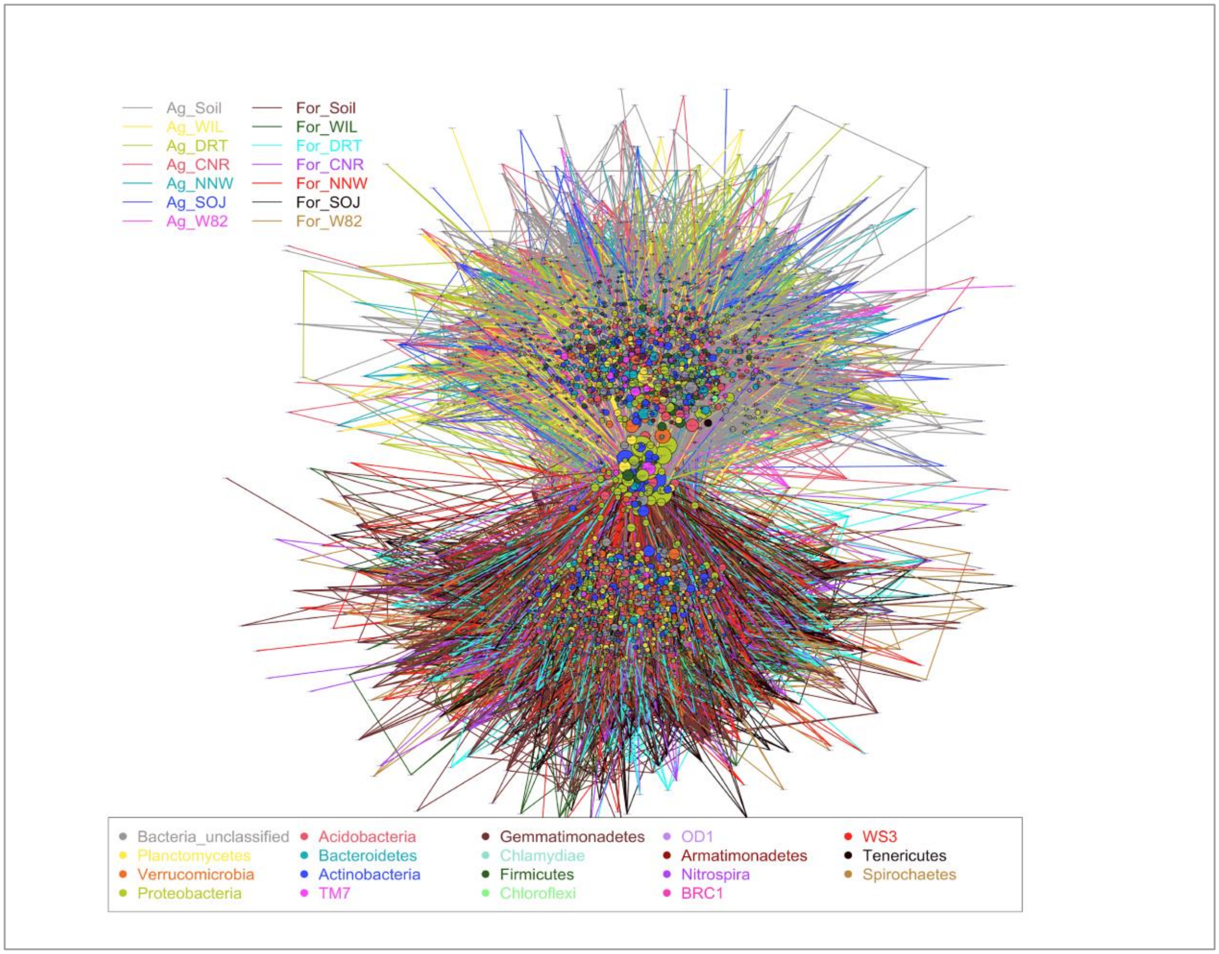


Fig. S2.6 Integrated microbial global network including all significant correlations between OTUs.

Inside of this network, each node represents one OTU and the correlations between OTUs were illustrated as edges. Nodes color were defined by their corresponding phylum, while edge color represents the treatment each edge belongs to. Whenever one edge was shared by several different treatments, the color was redefined by color mixer. Node size was represented by scaling the degree of connection of each node.

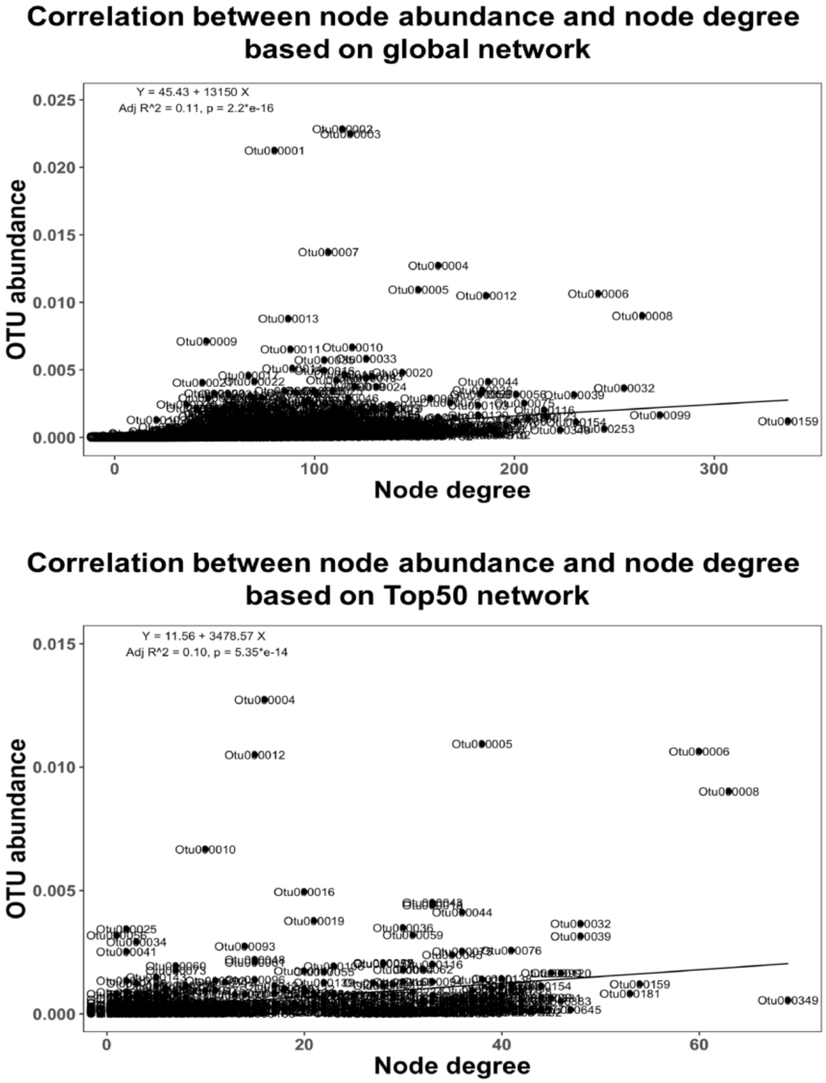


Fig. S2.7 Correlation analysis between network node degree and corresponding OTU relative abundance using both global network and Top50 network.

Each node was labeled with OTU_ID, whose taxonomy information could be found in Supplemental Table 3. Though p-value indicate significant correlation, only 10%-11% variance could be explained by this linear regression in both networks.

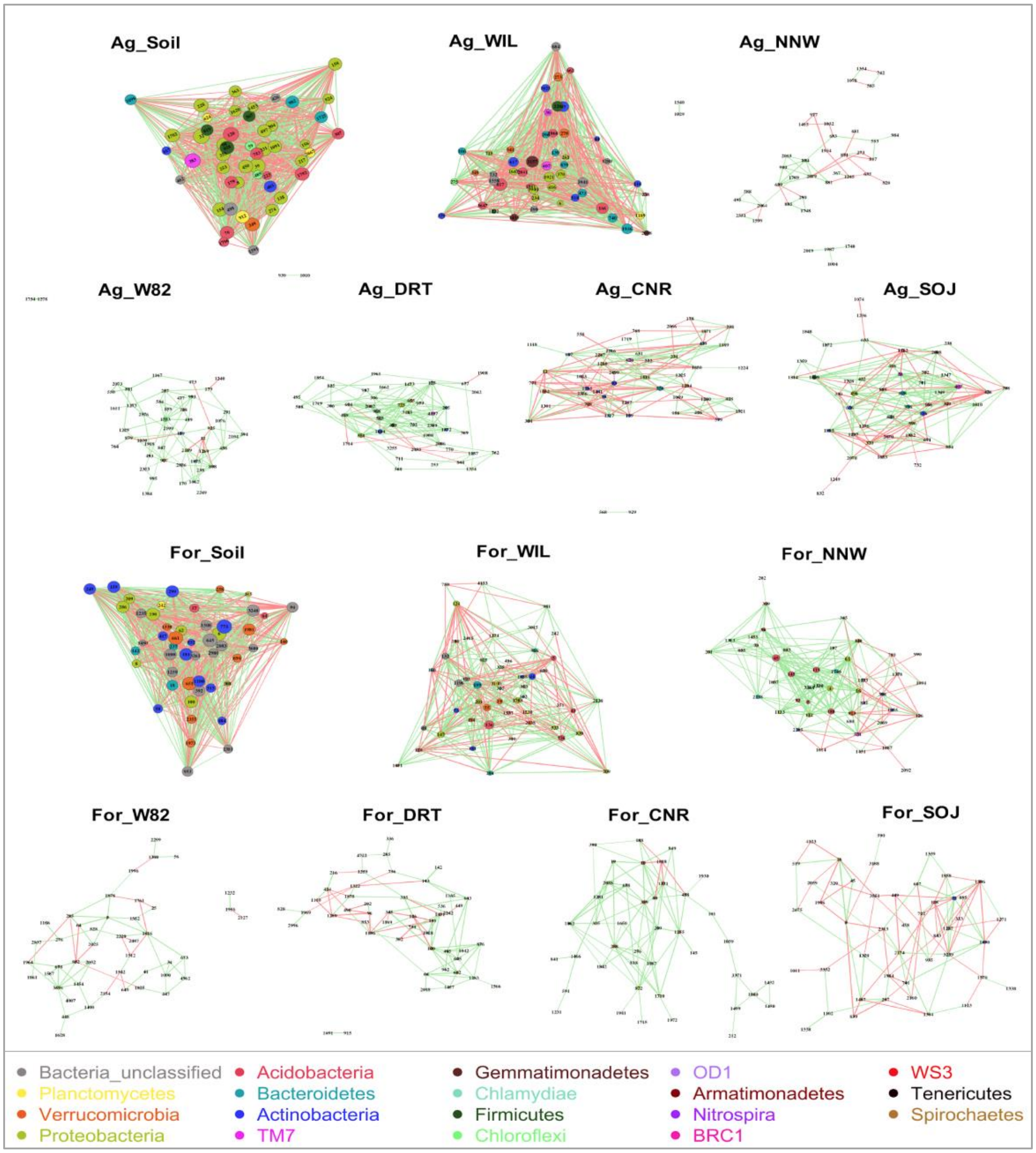


Fig. S2.8 Individual microbial network constructed using top 50 nodes in terms of connection degree.

In the below networks, nodes were colored based on phylum name as listed below. Red edge indicates negative correlation between OTUs while green edge represents positive correlation. Node size was defined based on connection degree. The bigger the node size, the more connections it has.

Table S2.1: GraPhlAn nodes taxonomy.xlsx (attached files).

Table S2.2: The soil type-dependent enrichment of bacterial genus and their relative abundance.xlsx (attached files).

Table S2.3: Taxonomic information of the OTU nodes within co-occurrence network.xlsx (attached files).

Chapter 3 Strigolactones discriminantly modulate soybean rhizosphere bacterial and fungal microbiome assembly

Declaration

The material presented in this chapter will be used for journal article publication.

Abstract

Strigolactones are a recently discovered class of carotenoid-derived plant hormones with a wide variety of functions, including acting as signaling molecules in the rhizosphere to promote arbuscular mycorrhizal fungi colonization and parasitic seed germination. To determine if strigolactones influence the recruitment of microbes to the rhizosphere, we compared both bacteria and fungi communities in response to the overexpression of genes involved in strigolactone biosynthesis (Max1) and signaling perception (D14 and Max2) in soybean (*Glycine max*). Based on amplicon sequencing, the strigolactone overexpression lines had altered soybean rhizosphere bacteria composition at both the community level and individual taxa level with genera including *Shinella* and *Bdellovibrio* consistently more abundant across all three gene treatments. The gene mechanism was also significant, with lines overexpressing genes involved in strigolactone biosynthesis (Max1) yielding a divergent bacterial community in comparison to those with altered strigolactone perception genes (D14 and Max2). While the overexpressed genes did not significantly impact the overall fungal community distribution, some individual taxa were altered in abundance. Max1 and D14 overexpression lines had significantly enriched levels of *Fusarium solani*. Co-occurrence analysis revealed more connected bacteria-bacteria networks but less connected fungi-fungi networks in the rhizosphere compared to bulk soil. When considering the most highly connected bacterial taxa in the networks, the overexpression of strigolactone significantly increased the negative bacteria-bacteria relationships for those taxa. The functional role of plant strigolactone biosynthesis and signaling pathways on bacteria and fungi communities in the rhizosphere confirmed

strigolactone’s importance in rhizosphere communication and microbial community structure.

3.1 Introduction

Plants have evolved intimate interactions with their associated microbiome for various functions, including nutrient uptake, stress tolerance, pathogen defense, and phenotypic plasticity, all facilitated by diverse plant growth-promoting bacteria (Bakker et al. 2018; Goh et al. 2013). Comprehensive and mechanistic understanding of plant microbiome assembly is crucial for optimizing beneficial plant-microbe interactions and maximizing the benefits offered by the plant microbiome for agricultural applications (Pieterse, de Jonge, and Berendsen 2016). The rhizosphere, the narrow region between the plant root and attached soil, is a dynamic and active interface characterized by intensive communication between the plant and microbes (Philippot et al. 2013). In this zone, plants actively and dynamically modulate the composition and function of microbes via root exudates, secondary metabolites or specific signaling pathways in response to changing soil biotic or abiotic stimuli (Zhalnina et al. 2018; Stringlis et al. 2018; Lebeis et al. 2015; Castrillo et al. 2017; Xu et al. 2018; Berendsen et al. 2018). For example, salicylic acid is a foliar defense phytohormone that was also found to influence the root microbiome by altering the colonization of specific bacteria families (Lebeis et al. 2015). Similarly, coumarin scopoletin (an iron-mobilizing phenolic compound) mediates rhizosphere microbiome assembly via its discriminant antimicrobial activity to pathogens versus growth-promoting rhizobacteria (Stringlis et al. 2018).

Strigolactones are a group of relatively recently discovered carotenoid-derived plant hormones and signaling molecules involved in plant-microbe interactions (Al-Babili and Bouwmeester 2015; Cook et al. 1966). The biosynthesis of strigolactones starts with the sequential action of a ß-carotene isomerase DWARF27 (D27) and two carotenoid cleavage dioxygenases (CCD7 and CCD8) to produce carlactone, which is the last common precursor for all strigolactones (Waters et al. 2017). Carlactones are catalyzed by the cytochrome P450 enzyme more axillary growth 1 (Max1) and subsequent uncharacterized enzymes to produce either canonical or non-canonical strigolactones (López-Ráez, Shirasu, and Foo 2017; Waters et al. 2017). Expression of genes involved in SLs biosynthesis is highest in plant roots (e.g, rice, Arabidopsis, sorghum and peas) and low or undetectable in other tissues (Ruyter-Spira et al. 2013; Al-Babili and Bouwmeester 2015; Yoneyama et al. 2007). The synthesized SLs function as plant hormones via downstream signaling transduction enabled by α/β-fold hydrolase D14 and F-box protein Max2 (Beveridge and Kyozuka 2010; Arite et al. 2009; Nelson et al. 2011). Simultaneously, strigolactones are exuded into the rhizosphere, however, transport mechanisms are not yet well understood with the only characterized ABC transporter (PDR1, first identified in *Petunia hybrida*) (Kretzschmar et al. 2012).

Strigolactones have multifunctional roles in plant development and plant-biota communication. Strigolactones act as *ex planta* signaling molecules after being exuded into the rhizosphere by modulating plant-plant and plant-microbe communications (López-Ráez, Shirasu, and Foo 2017). The presence of strigolactones in the rhizosphere acts as a signal for root colonization by symbiotic arbuscular mycorrhizal fungi. This signaling has been profiled in diverse plant species including *Lotus japonicus*, petunia, *s*orghum, tomato and Fabaceae plants (Akiyama, Matsuzaki, and Hayashi 2005; Besserer et al. 2006; Yoneyama et al. 2008; Kretzschmar et al. 2012). Strigolactones in the soil are perceived as germination stimulants for root parasitic plants of the family Orobanchaceae (Yoneyama et al. 2008). Strigolactones also function *in planta* as phytohormones regulating shoot branching, root morphology and architecture, secondary growth and leaf senescence across a wide array of plants, including *Arabidopsis*, rice, pea, and petunia (Al-Babili and Bouwmeester 2015). Strigolactones are involved in plant response to unfavorable environmental conditions including drought, salinity, nutrient deprivation and pathogen infection, with research in this area emerging from several plant species including *Arabidopsis*, tomato, lettuce, sorghum and *Lotus japonicus* (López-Ráez 2016; Van Ha et al. 2014; Visentin et al. 2016; Ruiz-Lozano et al. 2001). However, in terms of how strigolactones mediate plant stress responses, we still lack consistent conclusions, possibly due to the diverse chemical structures of molecules in the strigolactone family among investigated plants and the complex cross-talk between strigolactones and other phytohormones (Waters et al. 2017).

Strigolactones have recently been suggested as important modulators for legume nodulation. In both pea and alfalfa, *in vitro* assays using the synthetic strigolactone analogue $\mathrm{GR24}^{rac}$ increased the number of nodules per plant (Foo and Davies 2011; Soto et al. 2010). Further, reduced nodule numbers were detected in strigolactones synthesis mutants *ccd7* and *ccd8* in comparison to wild type pea, and this reduction of nodule formation could be rescued by $\mathrm{GR24}^{rac}$ to a similar level as wild type (Foo and Davies 2011; Foo et al. 2013). The reduction of pea nodules could not be attributed to changes of root traits or He (Foo et al. 2013). Unlike the impacts of strigolactones on arbuscular mycorrhizal fungi (AMF) colonization (e.g., inducing hyphae growth, branching and spore germination) (Akiyama, Matsuzaki, and Hayashi 2005; Besserer et al. 2008; Waters et al. 2017), the nodulation promotion by strigolactones cannot be attributed to their stimulatory effects on rhizobia growth or nod genes expression (Foo and Davies 2011; Soto et al. 2010; Moscatiello et al. 2010). Peláez-Vico et al. (2016) demonstrated that the application of a synthetic strigolactone analog enhances surface motility of *Sinorhizobium meliloti*, a Rhizobia of alfalfa (Peláez-Vico et al. 2016), providing a possible mechanism for strigolactones to help establish rhizobia in nodules. However, inconsistent reports regarding the influence of strigolactones on legume nodulation also exist. For example, the strigolactone response mutant *rms4* developed a similar number of nodules to wild type, which could be attributed to the fact that *rms4* is involved in both strigolactone and karrikins signaling pathways (Foo et al. 2013). De Cuyper et al (2015) reported a dose-dependent regulation of GR^{24} on *Medicago truncatula* nodule numbers, with positive effects when applied at low concentration and negative effects under high concentration (De Cuyper et al. 2015).

Considering the many functions of strigolactones in regulating plant growth and mediating plant-microbe interactions, strigolactones have potential agricultural applications. The most significant translational applications are currently focused on parasitic weed control by interrupting host strigolactone signaling to prevent parasitic seed germination (Dor, Yoneyama, et al. 2011) or by finding mimics of strigolactones that trigger parasitic seed suicidal germination (Kgosi et al. 2012). However, altering strigolactones to influence parasitic plant germination could have an indirect impact on plant-microbe interactions. As plant microbiomes function as a community and strigolactones already have demonstrated impacts on important fungal and bacterial species, a comprehensive and community-level understanding of the modulating role of strigolactones on root-associated microbes is needed (Andreo-Jimenez et al. 2015). To fill this gap, we built soybean overexpression constructs targeting the Max1, Max2 and D14 genes using a transient root transformation technique (Kereszt et al. 2007). Amplicon sequencing of 16S rRNA gene and ITS2 region were used to characterize the taxonomic composition of bacterial and fungal communities in response to the over-expression of strigolactone biosynthesis and signaling genes. Our results indicated that the over-expression of genes involved in strigolactone biosynthesis and signal perception significantly altered bacterial community composition in the soybean rhizosphere, while only minor impacts were detected for the fungal community.

3.2 Methods and materials

3.2.1 Candidate gene selection

Genes involved in strigolactone biosynthesis and perception in Arabidopsis were extracted from the TAIR database (Berardini et al. 2015). Corresponding homologs in *Glycine max* were retrieved from Phytozome (Goodstein et al. 2012). (Table 3.1 and Fig. 3.1). In total, 19 *Glycine max* genes were collected. The mRNA sequence of each homolog was downloaded from SoyBase (Grant et al. 2010) and homolog-specific primer sets were designed for qRT-PCR using *Primer3* (Untergasser et al. 2012) online tools. To ensure primers specificity, we conducted multiple sequence alignments of gene family members using *Clustal Omega*, then designed primers in sequence regions unique to each gene. To quantify the relative expression level for each gene, RNA was extracted from the root tissue of a *Williams 82* plant with Trizol reagent and subsequently purified using chloroform extraction. After DNase treatment, the DNA-free

RNA was used for one-step qRT-PCR (Power SYBR® Green RNA-to-CT™ 1-Step Kit) with ubiquitin as a constitutive reference.

3.2.2 Over-expression clone construction and hairy root transformation

To build *Williams* 82 overexpression constructs, soybean root cDNA was synthesized by Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase using an oligo-dT primer, and the coding sequence of the three target genes was cloned into *E. coli* pG$_2$RNAi2 vector using AscI and BamHI restriction enzymes (Fig. S3.1). Kanamycin-selected positive clones were picked for colony PCR and sequencing confirmation. Sequencing-confirmed plasmids were then transferred to competent *Agrobacterium rhizogenes* K599 using the freeze-thaw method following the Kereszt et al. (2007) protocol (Kereszt et al. 2007). Briefly, surface sterilized soybean seeds were paper germinated in a growth chamber at 26˚C in the dark for two days. After germination, healthy soybean seedlings were transplanted to autoclaved vermiculite medium, and grown under 16h light/8h dark at 30˚C/24˚C for another two days. At this stage, an unfolded green cotyledon emerged. Meanwhile, *Agrobacterium* suspensions with over-expression vectors were prepared and inoculated via syringe injection. Suspensions without overexpression vectors were also prepared to use as an experimental control. The inoculated soybean seedlings were kept in 12h light/12h dark at 28˚C/25˚C in a humid growth chamber for 3-5 days until the development of visible swelling at the injection site. At this stage, all soybean seedlings with well-developed swelling were transplanted to bigger pots (with the area of swelling buried by vermiculite) to grow for another 2 weeks until the formation of hairy roots with length around 10-15cm, which is long enough to support the growth of the seedlings. At this point, soybean seedlings were gently pulled out of the pot and attached vermiculite washed off. The primary roots were cut off from soybean seedlings while the remaining hairy roots were kept based on GFP marker screening using a fluorescence microscope. During the screening process, all non-transgenic roots were removed, leaving only transgenic hairy roots.

3.2.3 Transgenic soybean seedling growth and rhizosphere sample collection

Fresh soil was collected from the East Tennessee Research and Education Center Plant Science Unit just before transgenic hairy root screening. After field collection, the soil was immediately transported to the greenhouse. After removing roots and debris, the soil was well homogenized and allocated to pots (diameter = 20cm, height = 25cm). Once the transgenic screening was completed, all of the selected hairy root transgenic seedlings were transplanted into pots filled with fresh soil, and grown in the greenhouse until the flowering stage (16h light/8h dark at 30°C/20°C with a relative humidity of 60-80%). Pots with soil but no plants were also maintained in the same conditions, and the soil from these is from here on referred to as bulk soil. Each treatment (overexpression and control vectors) was started with 10 biological replicates. Both soybean seedlings and control pots were watered as needed, approximately every other day. Due to loss of plants and low quality of DNA extracts, we got 7 or 8 replicates left and used for statistically analysis after sequencing and sequences preprocess.

At the flowering stage, soybean rhizosphere soil samples were collected according to (Lundberg et al. 2012). Briefly, pots with soybean seedlings were put upside down into a surface sterilized metal tray, and soybean roots were gently separated from the soil block. Loosely attached soil was shaken off the soybean roots, and the roots with adherent soil were placed into phosphate buffer (per liter: 6.33 g of NaH_2PO_4.H2O, 16.5g of Na_2HPO_4.7H2O, 200 µl Silwet L-77). The tubes were vortexed at maximum speed for 30 s and the slurry was filtered through a 100-µm cell strainer. The soil pellet centrifuged from the slurry was collected as the rhizosphere soil sample. A similar amount of bulk soil was collected from the pots with no plant, and processed in parallel with the rhizosphere samples. All of the extracted soil samples were flash-frozen in liquid nitrogen and stored at -80°C before DNA extraction.

3.2.4 DNA extraction, library preparation and equencing

Soil DNA was extracted using the *MoBio* soil DNA isolation kit (Qiagen) following the protocol. We performed 16S rRNA gene-based bacteria profiling with Illumina Miseq 275bp paired-end sequencing, targeting the V3-V4 regions using forward primer 341F= 5′-CCTACGGGNGGCWGCAG-3′ and reverse primer 785R = 5′-GACTACHVGGGTATCTAATCC-3′ (Takahashi et al. 2014). Library preparation followed the Illumina 16S rRNA gene amplicon sequencing protocol. Briefly, PCR with a 16S rRNA gene specific primer with adapter overhangs (compatible with Nextera XT index) was used to amplify the 16S rRNA V3-V4 region with 2X KAPA HiFi HotStart ReadyMix with the following PCR cycle: 95°C for 3 minutes; 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes. The PCR product was then purified with AMPure XP beads. During the second step of PCR, Illumina dual indices were ligated to the PCR product from step one using the Nextera XT Index Kit with PCR cycle: 95°C for 3 minutes; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes. To eliminate the amplification of plant material (chloroplast and mitochondria), peptide nucleic acid (PNA), including anti-mitochondrial PNA (mPNA) 5′-GGCAAGTGTTCTTCGGA-3′ and the anti-plastid PNA (pPNA) 5′-GGCTCAACCCTGGACAG-3′ were used to block their elongation during the first step of PCR (Lundberg et al. 2013). ITS based fungi profiling was accomplished with MiSeq 250bp paired-end sequencing using 6 forward and 2 reverse primers at an equal concentration (Cregger et al. 2018), which are designed to allow better detection of *Chytridiomycota*, *Sebacinales*, *Glomeromycota*, *Sordariales*, *Stramenopila*, and *Archaearhizomycetes*. Library preparation also followed the Illumina 16S metagenomic sequencing library preparation protocol.

3.2.5 Sequence analysis

Mothur software was used to process 16S rRNA and ITS2 sequences, including quality control, assembly, alignment, chimera removal, OTU (operational taxonomic unit) clustering and taxa classification (Schloss et al. 2009). For 16S rRNA sequences, OTUs were clustered at 97 percent similarity using aligned sequences and classified against the SILVA 132 taxonomy reference database using a naive Bayesian classifier (Kozich et al. 2013; Wang et al. 2007). As sequence variation within the 16S rRNA gene V3-V4 region is not sufficient to distinguish non-Agrobacterium *Rhizobium* with our vector carrier, *Agrobacterium rhizogenes* K599, any sequences classified as *Rhizobium* were removed from downward OTU clustering and community analysis (Additional information on this OTU and its sequence levels in experimental samples is available in Supplemental File S3.1). For ITS2 sequences, OTUs were clustered at 97 percent similarity using unaligned sequences, which were taxonomically classified based on the UNITE v8 reference (Nilsson et al. 2019). For both 16S and ITS2, any sequence pairs (forward and reverse reads) with a mismatch within the primer region were removed before assembly. Chimera sequences were detected and discarded using the vsearch tool based on the UCHIME algorithm (Rognes et al. 2016; Edgar et al. 2011). Sequences that belonged to chloroplast, mitochondria, eukaryotes, and archaea were discarded before OTU clustering for 16S rRNA data. Similarly, non-fungal sequences, including unknown, *Alveolata*, *Amoebozoa*, *Apusozoa*, *Choanoflagellozoa*, *Chromista*, *Cryptista*, *Euglenozoa*, *Eukaryota*, *Incertae_sedis*, *Filasteriae*, *Glaucocystoplantae*, *Haptista*, *Heterolobosa*, *Ichthyosporia*, *Metazoa*, *Picozoa*, *Planomonada*, *Protista*, *Protozoa*, *Rhizaria*, *Rhodoplantae*, *Stramenopila*, *Viridiplantae* were removed before OTU clustering and classification. To alleviate the bias introduced by uneven sequencing depth, rarefaction at the minimum sequencing depth of 26,012 (16S rRNA) and 9,169 (ITS2) was used for normalization. OTUs represented by a single read were removed before subsequent microbial community analysis in R.

To compare the compositional differences of bacterial and fungal communities between treatments, beta diversity between samples were calculated based on Bray-Curtis distance and visualized by PCoA or CAP plot. Shannon alpha diversity metrics were compared between treatments using a Kruskal-Wallis test, followed by pairwise comparison using Dunnett’s test at the threshold p value < 0.05. Differential abundance of bacteria and fungi at the genus level was analyzed using LefSe (Segata et al. 2011). Tax4Fun2 was used to infer the functional capacity of bacterial communities based on taxonomic information (Wemheuer et al. 2018). The metabolic pathway profiles generated from Tax4Fun2 were used as input for DESeq2 to test the treatment effects on individual metabolic pathways (Love, Huber, and Anders 2014). KEGG pathways with differential abundance between treatments were extracted for visualization using pheatmap (Kolde 2015). Similarly, FUNGuild was used to summarize fungal functions and guilds between treatments (Nguyen et al. 2016). The relative abundance of predicted fungi guilds was compared between treatments either using one way ANOVA or Kruskal-Wallis test depending on the distribution of corresponding dependent variables. To profile microbe-microbe interactions, we calculated the co-occurrence network of both the bacterial and fungal communities using the SparCC algorithm and visualized the network topology using iGraph (Friedman and Alm 2012; Csardi and Nepusz 2006). The precision of the SparCC approach is sensitive to data sparsity (Weiss et al. 2016; Friedman and Alm 2012). To handle this, the rarefied OTU count table was subset to keep only the top 500 most commonly observed OTUs within each treatment for both bacterial and fungal communities. To eliminate the false positive rate of predicted correlations, the significance threshold was set to $\alpha < 0.001$ (Weiss et al. 2016). The global network for each treatment was generated based on all significant connections, while the top 50 most connected nodes from each treatment were selected for visualization (Timm et al. 2018).

3.3 Results

3.3.1 Soybean over-expression constructs

To select candidate genes for transformation, we targeted highly expressed genes from the most downstream steps of the strigolactone synthesizing and sensing pathways (Xie, Yoneyama, and Yoneyama 2010; Ruyter-Spira et al. 2013) (Fig. 3.1). qRT-PCR results of 19 strigolactone-associated genes detected expression in root tissue for all

(Fig. 3.2A). The most highly expressed homologs of Max1, D14, and Max2 genes were selected (*i.e.*, Glyma.14g096900, Glyma.17g235300, and Glyma.12g128600) as targets for overexpression vector development and then, using the hairy root transformation method, transformed into the roots of ‘Williams 82’ soybean. As a control, a vector with only the GFP reporter was also used for transformation. Nine biological replicates were used for each gene and the control transformations. Screening the roots from transformed plants for the GFP reporter protein indicated that the overexpression vector was successfully transferred and expressed in the hairy roots of all individuals (Fig. 3.2B).

3.3.2 Microbial community composition and structure in the soybean rhizosphere

A total of 1,810,039 16S rRNA and 971,362 ITS2 amplicon sequences were retained after initial trimming and screening with *Mothur* software (Schloss et al. 2009). These sequences were clustered into 42,313 bacterial OTUs and 4,634 fungal OTUs across 36 samples (7 Max1, 7 D14, 7 Max2, 7 empty construct controls, 8 bulk soil). After rarefaction and removal of singletons, 12,368 bacterial OTUs and 1,607 fungal OTUs remained, which were classified into 1,048 genera and 750 species respectively (Table S3.1). Rarefaction plots indicated consistent sampling of taxa across individual libraries and sufficient sequencing depth for the fungal community, however, species richness was likely not fully sampled for the bacterial community (Fig. S3.2).

In the soybean rhizosphere and bulk soil, the bacterial community was dominated by *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Acidobacteria*, *Verrucomicrobia*, *Planctomycetes,* and *Chloroflexi*, which altogether accounted for about 90% of total bacterial abundance (Fig. 3.3A). Compared with the bulk soil bacterial community, *Alphaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, and *Firmicutes* were significantly enriched in the soybean rhizosphere (Fig. 3.3B) while the relative abundance of *Deltaproteobacteria*, *Acidobacteria*, *Chloroflexi*, and *Planctomycetes* was significantly reduced ($p < 0.05$). The abundance difference of the *Bacteroidetes* phylum between bulk soil and soybean rhizosphere was not significant. Regarding the fungal community, both the bulk soil and the soybean rhizosphere were dominated by *Sordariomycetes*, *Dothideomycetes*, *Mortierellomycetes* and *Agaricomycetes* classes (Fig. 3.4A). In the soybean rhizosphere, *Glomeromycetes, Entomophthoromycetes*, and *Eurotiomycetes* were significantly enriched compared with bulk soil, while *Mucoromycetes* and *Mortierellomycetes* were significantly depleted ($p < 0.05$) (Fig. 3.4B).

3.3.3 Rhizosphere bacterial composition and diversity across treatments

The taxonomic composition of the bacterial community differs significantly between the bulk soil and the soybean rhizosphere (Fig. 3.5A). PERMANOVA results showed that 27.38% of the bacteria composition variation could be explained by the compartment difference (PERMANOVA, $F_{(1,34)} = 12.82$, $p < 0.001$). The overexpression impacts of MAX1, D14 and MAX2 were also significant as reflected by the clear separation of control and over-expression samples in a CAP plot (Capscale, $F_{(3,24)} = 1.56$, $p = 0.007$) (Fig. 3.5B). The composition of the bacterial community also differs between the overexpression gene treatments. Rhizosphere microbial communities from the plants overexpressing the strigolactone perception genes D14 and MAX2 were very similar to each other and slightly but significantly diverged in taxonomic composition from the rhizosphere communities of plants overexpressing MAX1, a strigolactone biosynthesis gene (Capscale, $F_{(2,18)} = 1.48$, $p = 0.039$). The impact of sequencing depth was also evaluated and found to be significant, however, it explained only 6.8% of the community differences between treatments (PERMANOVA, $F_{(1,34)} = 2.50$, $p = 0.017$). The Shannon index of alpha diversity of the bacterial community in bulk soil was different from that of rhizosphere microbiome, with a significant reduction of alpha diversity in non-transgenic control and MAX1 overexpression constructs compared with bulk soil (Kruskal-Wallis chi-squared = 16.981, df = 4, p = 0.002) (Fig. 3.6A). However, no significant difference in bacterial diversity was detected between D14, MAX2 and bulk soil.

The *Microbacteriaceae* family, *Rhizobiaceae* family (especially *Shinella genus*) and *Bdellovibrionaceae* family (especially *Bdellovibrio* genus) were consistently enriched across overexpression constructs compared with the control (Fig. 3.7 and Fig. S3.3 and Table S3.2). In examining each treatment against the control, we found different enriched/depleted bacteria taxa: MAX1 (10 enriched/1 depleted), D14 (15 enriched/6 depleted), MAX2 (36 enriched/8 depleted). The overall pattern suggests the overexpression of strigolactone perception genes impacted the abundance of more taxa compared to strigolactone biosynthesis genes. For example, *Deltaproteobacteria* and *Dyadobacter* were significantly enriched in D14 and MAX2 overexpression constructs compared with control soybeans while *Gammaproteobacteria* (*Pseudomonas* specifically) was significantly depleted (Fig. 3.7 and Fig. S3.3). However, none of these three were detectable for the MAX1 overexpression construct.

3.3.4 Rhizosphere fungal composition and diversity across treatments

Similarly to the bacterial community, the composition of the fungal community differs significantly between the bulk soil and soybean rhizosphere, explaining 13.16% of community variation ($F_{(1,34)} = 5.15$, $p < 0.001$) (Fig. 3.5C). However, detection of a significant difference between control soybean samples and those with overexpression constructs was not significant, with the significance statistic meeting a relaxed p-value cutoff of 0.01, but not a more stringent $p < 0.05$ cutoff (Capscale, $F_{(3,24)} = 1.22$, $p = 0.097$) (Fig. 3.5D). Significant differences were not detected between MAX1, D14 and MAX2 overexpression constructs (Capscale, $F_{(2,18)} = 1.07$, $p = 0.306$). The sequencing depth was a significant factor (PERMANOVA, $F_{(1,34)} = 2.97$, $p = 0.003$), explaining 8% of the community composition variations between samples.

In terms of fungal community diversity, the Shannon diversity index was similar between the bulk soil and soybean rhizosphere as well as between the soybean control construct and over-expression constructs (Kruskal-Wallis chi-squared = 6.53, $p = 0.16$) (Fig. 3.6B). Unlike the separation of bacterial community composition between biosynthesis gene overexpression (Max1) and perception gene overexpression (D14 and Max2), the fungal community composition for MAX1 and D14 were more similar to each other than to MAX2 (Fig. S3.4). Despite the lack of significant differences in overall community composition, individual taxa were significantly enriched/depleted by the overexpression of strigolactones. Specifically, *Fusarium solani* was consistently enriched in the soybean rhizosphere with the overexpression construct of MAX1 and D14 when compared to the control but not for MAX2 in comparison to the control (Fig. 3.7). In contrast, the *Rhizophlyctidales* order was selectively enriched to a higher degree in MAX2 overexpression construct compared to control, while *Spizellomycetales* and *Sordariales* order were less abundant compared with control soybeans. Surprisingly, the symbiotic arbuscular fungi, *Glomeraceae,* was not significantly different in abundance between control and all overexpression constructs.

3.3.5 Microbe-microbe interaction and function difference in soybean rhizosphere and between treatments

Microbe-microbe interactions are crucial factors structuring plant microbiome assembly and function (Agler et al. 2016; Hassani et al. 2018). We constructed co-occurrence networks for both bacterial and fungal communities to infer the changes of microbe-microbe interactions in response to the overexpression of strigolactone-associated genes. For the bacterial community, the network was more complex and had higher edge density in the soybean rhizosphere compared to bulk soil (Table 3.2). To understand the overlaps of highly connected “hub” OTUs between treatments, the top 50 most connected OTUs from each treatment were selected and merged into a united network.

The bacterial networks differed between treatments as reflected by the distinct subnetworks for each treatment (Fig. 3.8A and Table S3.3). The OTU nodes of this united network mainly belong to *Acidobacteria* (17.53%), *Proteobacteria* (15.46%), *Actinobacteria* (14.43%), *Planctomycetes* (13.40%) and *Bacteroidetes* (12.89%). Taxa belonging to *Acidobacteria Gp6* (i.e., OTU00036, OTU00073, OTU00105, OTU00149, and OTU00170), *Agromyces* (i.e., OTU00030), *Glycomyces* (i.e., OTU00007), *Dongia* (i.e., OTU00051) and *RB41* (i.e., OTU00061) were in the top 50 taxa for each treatment and also function as the connecting nodes between treatment, suggesting they play crucial roles in bacterial network structure. The co-occurrence network of bacteria in the bulk soil was much simpler, as indicated by the lowest average node degree and edge density compared with that of rhizosphere samples despite the higher bacterial diversity in bulk soil (Table 3.2 and Fig. S3.5). Interestingly, the ratio of negative bacteria-bacteria correlations was consistently increased in strigolactone overexpression constructs in comparison to control treatment.

Compared with the bacterial community network, the fungal co-occurrence networks were much simpler and featured very sparse interactions between taxa (Fig. 3.8B and Table S3.4). The taxa in the network were dominated by *Ascomycota* and *Basidiomycota*. The fungal occurrence network from bulk soil was more complex than the rhizosphere microbiome networks, in a pattern opposite to the bacterial community (Table 3.2 and Fig. S3.6). Interestingly, the percentage of positive fungi-fungi interactions also decreased in the rhizosphere of overexpression constructs in comparison to non-transgenic control. For both fungal and bacterial networks, the network edge density was consistently higher in D14 overexpression constructs compared with that of MAX1, MAX2 and control treatments. When we compare the bacterial network topological features from global perspectives (the network including all significant interactions), the overall pattern between treatments was similar; however, the ratio of positive microbe-microbe interactions of global networks differed from Top50 based network. At the global level (before subsetting to the 50 most connected taxa), SparCC gave a more balanced distribution of positive and negative interactions; when the most connected taxa were selected, the ratio of positive interactions increased.

Functional prediction with Tax4Fun revealed distinct functional capacities between bulk soil and the soybean rhizosphere bacterial communities (Fig. 3.9A). The phosphotransferase system, fructose, and mannose metabolism, and other glycan degradation metabolic pathways were significantly enriched in the soybean rhizosphere. In contrast, xenobiotic degradation related pathways, including polycyclic aromatic hydrocarbon, furfural degradation, aminobenzoate degradation were significantly decreased in the soybean rhizosphere. However, we did not see significant differences between the control and overexpression constructs despite the distinct bacterial community compositions among them.

FUNGuild was applied to link fungal taxa information with fungi ecological guilds. As some fungi play different ecological roles depending on their life history and surrounding environmental conditions, some of the fungal taxa could not be uniquely assigned to the specific trophic mode or guilds (Nguyen et al. 2016). In our study, 680 out of 750 species were assigned into seven different trophic modes after removing all undefined taxa data (ie., 15-31%) (Fig 3.9B). As expected from soil samples, the fungal community included mainly pathotrophs and saprotrophs with fewer being assigned to a symbiotroph mode. Multi-trophic assignments were common, with a high proportion (around 50%) of PS (pathotroph-saprotrophs) and PSM (pathotroph-saprotroph-symbiotrophs) within the fungi community. Overall, the trophic modes composition of the fungal communities differed only slightly between bulk soil and rhizosphere as well as among overexpression treatments. Statistically, saprotrophs were significantly decreased in MAX2 constructs and symbiotrophs were significantly reduced in MAX1 and D14 constructs in comparison with the control treatment (Fig. 3.9B).

3.4 Discussion

In this study, we modified the expression of soybean (Williams 82) strigolactone biosynthesis and perception genes using a hairy root transformation approach (Kereszt et al. 2007) and investigated the differences of soybean rhizosphere bacteria and fungi communities between overexpression constructs and the soybean control lines. We chose to utilize the hairy root transformation technique as an efficient method to impact the expression of target genes specifically in the roots. Our initial results indicate that altered expression of strigolactone-related genes influences bacterial community assembly in the rhizosphere of soybean, warranting future studies utilizing stable transgenics with *in planta* and *ex planta* quantification of strigolactone exudate levels.

3.4.1 Bacterial and fungal communities in the soybean rhizosphere versus bulk soil

The rhizosphere is one of the most dynamic interfaces between the soil and plant roots with a specialized microbial community that influences plant activities (Philippot et al. 2013). Plants exude numerous compounds, including up to 21% of their fixed carbon, into the rhizosphere to support the activity of associated microbes in exchange for growth promoting benefits (Lugtenberg and Kamilova 2009; Marschner and Marschner 1995). The root exudate profile dynamically drives the rhizosphere microbiome composition through signaling compounds and attracting microbes with specific substrate preferences (Zhalnina et al. 2018). The experiments reported here agreed with former reports about the compositional and functional differences of the microbial community between the soybean rhizosphere and bulk soil (Mendes et al. 2014; Zhang et al. 2018; Liu et al. 2019) with bacteria belonging to *Gammaproteobacteria* and

Actinobacteria significantly enriched in the soybean rhizosphere while bacteria of *Acidobacteria*, *Chloroflexi* and *Planctomycetes* were significantly depleted. This selective enrichment or depletion pattern in the soybean rhizosphere is consistent with the pattern that enriched nutrients in the plant rhizosphere tend to enhance the growth of copiotrophs, e.g., *Actinobacteria*, *Betaproteobacteria*, *Alphaproteobacteria* and *Firmicutes*, while oligotrophs including *Acidobacteria*, *Verrucomicrobia* and *Chloroflexi* are less competitive in this labile carbon enriched habitat (Pérez-Jaramillo, Mendes, and Raaijmakers 2016; Fierer, Bradford, and Jackson 2007; Ho, Di Lonardo, and Bodelier 2017; Eilers et al. 2010).

In terms of the fungal community, the composition of both bulk soil and the soybean rhizosphere was dominated by *Sordariomycetes*, *Dothideomycetes*, *Mortierellomycetes*, and *Agaricomycetes* classes. These fungi have been commonly found in soil and plant rhizospheres although their relative abundance differs between studies (Urbina et al. 2018; Coleman-Derr et al. 2016; Singer et al. 2019; Han et al. 2017). In this experiment, the selection of fungal taxa in the soybean rhizosphere was found to be insignificant at the phylum level but significant at lower taxonomic levels as revealed by the differential abundance analysis. Rhizosphere versus bulk soil differences was less evident for the fungi community with 13.16% explained variation compared with the bacteria community with 27.38% explained variation. This is in accordance with the view that fungi have more oligotrophic features compared with bacteria which may make fungi, in general, less responsive to the increased labile carbon resource of rhizodeposits (Ho, Di Lonardo, and Bodelier 2017).

Arbuscular mycorrhizal fungi (AMF) are known to colonize 80% terrestrial plants, including soybean as an important legume crop (Jones 1924; Smith and Read 2010). The *Glomeromycetes* class*,* which include all the AMF species (Lee et al. 2013), was found to be enriched the soybean rhizosphere. The symbiotic AMF function as natural fertilizers by expanding the nutrient accessibility and stimulating the decomposition of recalcitrant organic matter via synergistic interaction with bacteria (Hodge 2001; Artursson, Finlay, and Jansson 2006; Williams et al. 2017; Smith and Read 2010). The enrichment of *Glomeromycetes* in the soybean rhizosphere may reflect the importance of first recruiting AMF into the rhizosphere in order to establish the symbiotic interaction between soybean and AMF. AMF may also have a direct role in the rhizosphere; the hyphae of AMF were revealed to increase soil aggregate stability by excreting glomalin, which may help maintain good water infiltration rates, good tilth and adequate aeration for plant growth (Caravaca et al. 2006; Wright and Upadhyaya 1998). The enrichment of AMF in the soybean rhizosphere could potentially help maintain a stable soil physical structure for more efficient nutrient and water absorption.

3.4.2 Overexpression of strigolactone-associated genes modified soybean rhizosphere bacterial community

In this study, overexpression of strigolactone biosynthesis and signaling genes in soybean plants had significant impacts on rhizosphere bacteria assembly with distinct bacterial community compositions between the MAX1, D14 and MAX2 overexpression constructs and the control. Considering the multifaceted functions of strigolactone both *in planta* and *ex planta* (López-Ráez, Shirasu, and Foo 2017), this impact on rhizosphere bacterial community could be elicited directly by acting as a signaling molecule after exudation into the rhizosphere or indirectly by its modification on soybean root morphology or via cross-talk with other plant hormones (Cheng, Ruyter-Spira, and Bouwmeester 2013). Alternatively, strigolactones could be utilized by bacteria as a specific preferred carbon source. However, strigolactones are exuded at the nano and picomolar quantities outside of the plant and tend to decompose rapidly (Xie, Yoneyama, and Yoneyama 2010), making their primary impact as a carbon source less likely. More intriguingly, the bacterial composition of MAX2 and D14, both genes involved in strigolactone perception, were quite similar to each other and differed from MAX1, a gene involved in strigolactone biosynthesis. Further research is needed to understand how strigolactone perception within the soybean plant may play a critical role in mediating soybean rhizosphere bacteria assembly through altering strigolactone production or exudation levels. It is worth noting that MAX2 participates in both strigolactones and karrikin (KAR, a water soluble compound discovered in smoke) signaling pathways, with the latter pathway being revealed to trigger seed germination after fires/burning event (Smith and Li 2014; Nelson et al. 2011; Flematti et al. 2009). The distinct differences of rhizosphere bacteria between MAX1 and MAX2 overexpression plants, which is less evident between MAX1 and D14 constructs, may reflect the dual role of the MAX2 gene in both strigolactones and KAR signaling pathway (Nelson et al. 2011).

Recent studies applying gene manipulation and GR24 applications suggest strigolactones impact legume nodulation (Foo and Davies 2011; Foo et al. 2013; Peláez-Vico et al. 2016; De Cuyper et al. 2015) A comprehensive investigation of the strigolactone signaling pathway impacts on the nodulation of pea concluded that strigolactone manipulation on nodulation was mediated by its promotion of infection thread formation (McAdam et al. 2017). However, considering the complex microbe-microbe interactions in the soybean rhizosphere and within soybean nodules (Martínez-Hidalgo and Hirsch 2017), the fundamental role of strigolactone on rhizobia-legume interaction could benefit from an additional investigation from a global bacterial community perspective.

The differential abundance analysis from this study revealed stronger recruitment of *Rhizobiaceae* from bulk soil to the soybean rhizosphere in response to overexpression of strigolactone biosynthesis and perception genes, suggesting strigolactones as a mechanism to selectively recruit microbes to the rhizosphere prior to the establishment in nodules (Bulgarelli et al. 2013). We also detected significant enrichment of the specific genus *Rhizobium* in the rhizosphere of overexpression treatment groups in comparison to the control. However, we were not able to distinguish nitrogen-fixing rhizobia from *Agrobacterium rhizogenes* (the delivery agent of the overexpression constructs) due to the insufficient resolution of 16S rRNA sequencing. Regarding the potential impacts of strigolactone on rhizobia enrichment in the rhizosphere, no significant abundance changes between control and treatments were detected for *Bradyrhizobium* and *Sinorhizobium*, the most predominant nodule-formation rhizobia of soybean (Lindström et al. 2010). This is consistent with McAdam et al. (2017), which found that the deficiency of strigolactone biosynthesis gene CCD8 did not change the exudation of flavonoids, which function as a chemo-attractant to recruit *Bradyrhizobium japonicum* (Graham 1991; Khan and Bauer 1988).

Our experiment found the bacteria genera *Shinella* and *Bdellovibrio* were significantly enriched across overexpression constructs. One *Shinella* strain, *Shinella kummerowiae,* was isolated from the herbal legume *Kummerowia stipulaceae* nodules and found to have nodulation and nitrogen-fixing genes (Lin et al. 2008), while other *Shinella* species have been repeatedly found in contaminated or waste water functioning as nitrogen-reduction or sulfolane assimilating bacteria (Mu et al. 2018; Vaz-Moreira et al. 2010). The enrichment of the *Shinella* genus in response to overexpression of strigolactone could belong to any aforementioned strains, suggesting total DNA sequencing as the next approach for species-level identification. The *Bdellovibrio* genus includes bacteria able to parasitize and kill other bacteria. *B. bacteriovorus* has been reported as a predatory bacteria of soybean bacterial blight disease and has been proposed as a potential biocontrol for other phytopathogens (Scherff 1973; Olanya and Lakshman 2015). We detected intensive negative microbe-microbe interaction between Bdellovibrio and other bacteria taxa, further supporting the role of *Bdellovibrio* as a pathogen of other bacterial species.

3.4.3 Insignificant impact of strigolactone overexpression on metabolic functions of rhizosphere bacteria

Plant associated bacteria are adapted to their host plant environment and are enriched with genes involved in carbohydrate metabolism (Levy et al. 2018). In this study, we predicted bacterial metabolic pathways based on 16S rRNA data. Our results revealed a specialized metabolism potential in the soybean rhizosphere compared with that of bulk soil, with fructose and mannose metabolism enriched across samples in the soybean rhizosphere. Other glycan degradation pathways were also highly accumulated in the soybean rhizosphere. This is consistent with other reports, which found an enhanced carbohydrate metabolism in plant rhizosphere in response to the abundant root exudates available in the rhizosphere (Lu et al. 2018; Bulgarelli et al. 2015). The phosphotransferase system (PTS) is also an important factor for chemotaxis toward carbohydrates, pathogen virulence, bacterial cellular mobility and bacteria stress response (Neumann, Grosse, and Sourjik 2012; Deutscher et al. 2008; Barabote and Saier 2005). The enhanced PTS pathway found in the soybean rhizosphere is in accordance with the microenvironment, where abundant carbon resources, as well as antimicrobial secondary metabolites, are supplied by the host plant via root exudates (Baetz and Martinoia 2014). In this study, we did not observe a significant impact of the overexpression of strigolactone genes on predicted rhizosphere bacterial metabolic pathways despite significant alteration of bacteria composition. This inconsistency between composition and function response could be explained by the functional redundancy between bacteria taxa. Alternatively, this discrepancy could be caused by a lack of functional annotation for many soil bacterial species.

3.4.4 Strigolactone overexpression impacts individual fungal taxa but is insignificant at the community level

After examining the fungi compositional response to strigolactone overexpression, we did not observe significant changes at the community level. This is in contrast to Carvalhais et al. (2019), who disrupted CCD8/MAX4 in Arabidopsis and found significant modulation of the rhizosphere fungi community (Carvalhais et al. 2019). Strigolactones are widely recognized as a triggering signal for arbuscular mycorrhizal fungi hyphae growth and activities (Akiyama, Matsuzaki, and Hayashi 2005; Besserer et al. 2008), and disrupting gene expression may have a larger community impact than our approach, to overexpress genes. FUNGuild predicted 4% of fungi in the soybean rhizosphere as mycorrhizal. The surprisingly low abundance of mycorrhizal fungi was also reported in a poplar root microbiome study (Cregger et al. 2018). The surprisingly low abundance of mycorrhizal fungi could be due to some fungal endophytes shifting their ecological strategy from an endophytic to a saprophytic phenotype (Promputtha et al. 2007). This possibility is supported by a large percent of fungi in the soybean rhizosphere being predicted as pathotroph-symbiotroph (PS).

At the individual taxa level, we found significant and consistent enrichment of *Fusarium solani* species in MAX1 and D14 but not MAX2 overexpression constructs. There are numerous reports about strigolactones inhibition to fungal pathogen disease based on both gene mutation and GR^{24} assays (Torres-Vera et al. 2014; Dor, Joel, et al. 2011; Belmondo et al. 2017) others declared no impacts of strigolactones on fungal disease development (Foo et al. 2016; Blake et al. 2016). *Fusarium solani (Mart.) Sacc. f. sp. glycines* strain is a fungal pathogen causing sudden death syndrome, which was ranked as one of the most devastating soybean diseases across the top 10 major soybean growing areas (Roy 1997; Wrather et al. 2001). Although ITS2 sequencing based results were not sufficient to claim whether these enriched *Fusarium solani* are pathogenic or not, this enrichment of *Fusarium solani* in response to MAX1 and D14 gene expression warrants future investigation.

3.4.5 Strigolactone impact on bacteria and fungi co-occurrence network

In addition to plant-microbe interactions, microbe-microbe interactions are critical driving forces during microbial community assembly. Microbial interactions may be more important than environmental variables in determining community structure and plant health (Zhang et al. 2018; Wei et al. 2015). Correlation-based network analysis is a powerful tool to generate hypotheses and visualize interaction patterns between taxa, with positive correlations indicating mutualistic interactions and negative correlations antagonistic interactions (Weiss et al. 2016). In this study, microbial co-occurrence networks were built with the SparCC algorithm, which was designed to infer correlation with higher accuracy by considering compositional artifacts from amplicon sequencing data (Friedman and Alm 2012).

In this study, we observed distinct network structure and topological feature differences between bulk soil and soybean rhizosphere for both bacterial and fungi communities. The network density and complexity of the bacterial community dramatically increased in the soybean rhizosphere, which was consistent with other studies (Shi et al. 2016; Yan et al. 2017). These topological changes of the bacterial network may reflect more active interactions between microbes or more convergent responses to the enriched nutrient in the rhizosphere habitat (Shi et al. 2016; Weiss et al. 2016). However, the opposite trend was observed for fungal networks, which may reflect distinct drivers for fungal community structure in comparison to that of bacteria. Alternatively, this distinct trend potentially indicates divergent physiological and ecological adaptations of fungi to the rhizosphere niche considering the fact that correlation-based networks could either indicate direct interaction or mirror consistent response of taxa to specific environment traits (Weiss et al. 2016).

Intriguingly, when examining the most connected microbes, negative correlations were highly enriched for both bacteria and fungi community under the overexpression of strigolactone compared to the control. This increased negative interactions between taxa may indicate intensified antagonistic relationships such as competition between those actively connected taxa in response to overexpression of strigolactone. However, this network shift was not evident when examined at the global level. Networks are generally highly sensitive but suffer from low specificity, especially with low sample numbers, thus giving confidence in the relationships detected but making it difficult to compare specific co-occurrence relationships between treatments due to likely high numbers of undetected relationships (Shizuka and Farine 2016).

3.5 Conclusions and perspectives

Plant secondary metabolites, especially those involved in plant defense response, are increasingly being reported as crucial mechanisms that mediate root microbiome assembly. While the plant hormones strigolactones are known to influence plant-fungi interactions, particularly AMF, we demonstrate that the strigolactone signaling and biosynthesis pathway alters the rhizosphere bacteria community as well. For both fungi and bacteria, we demonstrate microbial alterations in the rhizosphere in terms of community composition, structure and potential function. Increased occurrence of taxa such as *Rhizobiaceae*, *Shinella*, *Bdevorella*, and *Fusarium solani* indicate this pathway may be important for signaling of microbes involved in recruit specific taxa for nodulation and nitrogen fixation as well as influencing microbe-microbe interactions and pathogens.

Reference

Agler, Matthew T., Jonas Ruhe, Samuel Kroll, Constanze Morhenn, Sang-Tae Kim, Detlef Weigel, and Eric M. Kemen. 2016. “Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation.” PLoS Biology 14 (1): e1002352.

Akiyama, Kohki, Ken-Ichi Matsuzaki, and Hideo Hayashi. 2005. “Plant Sesquiterpenes Induce Hyphal Branching in Arbuscular Mycorrhizal Fungi.” Nature 435 (7043): 824–27.

Al-Babili, Salim, and Harro J. Bouwmeester. 2015. “Strigolactones, a Novel Carotenoid-Derived Plant Hormone.” Annual Review of Plant Biology 66 (January): 161–86.

Andreo-Jimenez, Beatriz, Carolien Ruyter-Spira, Harro J. Bouwmeester, and Juan A. Lopez-Raez. 2015. “Ecological Relevance of Strigolactones in Nutrient Uptake and Other Abiotic Stresses, and in Plant-Microbe Interactions Below-Ground.” Plant and Soil 394 (1): 1–19.

Arite, Tomotsugu, Mikihisa Umehara, Shinji Ishikawa, Atsushi Hanada, Masahiko Maekawa, Shinjiro Yamaguchi, and Junko Kyozuka. 2009. “d14, a Strigolactone-Insensitive Mutant of Rice, Shows an Accelerated Outgrowth of Tillers.” Plant & Cell Physiology 50 (8): 1416–24.

Artursson, Veronica, Roger D. Finlay, and Janet K. Jansson. 2006. “Interactions between Arbuscular Mycorrhizal Fungi and Bacteria and Their Potential for Stimulating Plant Growth.” Environmental Microbiology 8 (1): 1–10.

Baetz, Ulrike, and Enrico Martinoia. 2014. “Root Exudates: The Hidden Part of Plant Defense.” Trends in Plant Science 19 (2): 90–98.

Bakker, Peter A. H. M., Corné M. J. Pieterse, Ronnie de Jonge, and Roeland L. Berendsen. 2018. “The Soil-Borne Legacy.” Cell 172 (6): 1178–80.

Barabote, Ravi D., and Milton H. Saier Jr. 2005. “Comparative Genomic Analyses of the Bacterial Phosphotransferase System.” Microbiology and Molecular Biology Reviews: MMBR 69 (4): 608–34.

Belmondo, S., R. Marschall, P. Tudzynski, J. A. López Ráez, E. Artuso, C. Prandi, and L. Lanfranco. 2017. “Identification of Genes Involved in Fungal Responses to Strigolactones Using Mutants from Fungal Pathogens.” Current Genetics 63 (2): 201–13.

Berardini, Tanya Z., Leonore Reiser, Donghui Li, Yarik Mezheritsky, Robert Muller, Emily Strait, and Eva Huala. 2015. “The Arabidopsis Information Resource: Making and Mining the ‘gold Standard’ Annotated Reference Plant Genome.” Genesis 53 (8): 474–85.

Berendsen, Roeland L., Gilles Vismans, Ke Yu, Yang Song, Ronnie de Jonge, Wilco P. Burgman, Mette Burmølle, Jakob Herschend, Peter A. H. M. Bakker, and Corné M. J. Pieterse. 2018. “Disease-Induced Assemblage of a Plant-Beneficial Bacterial Consortium.” The ISME Journal 12 (6): 1496–1507.

Besserer, Arnaud, Guillaume Bécard, Alain Jauneau, Christophe Roux, and Nathalie

Séjalon-Delmas. 2008. “GR24, a Synthetic Analog of Strigolactones, Stimulates the Mitosis and Growth of the Arbuscular Mycorrhizal Fungus Gigaspora Rosea by Boosting Its Energy Metabolism.” Plant Physiology 148 (1): 402–13.

Besserer, Arnaud, Virginie Puech-Pagès, Patrick Kiefer, Victoria Gomez-Roldan, Alain Jauneau, Sébastien Roy, Jean-Charles Portais, Christophe Roux, Guillaume Bécard, and Nathalie Séjalon-Delmas. 2006. “Strigolactones Stimulate Arbuscular Mycorrhizal Fungi by Activating Mitochondria.” PLoS Biology 4 (7): e226.

Beveridge, Christine Anne, and Junko Kyozuka. 2010. “New Genes in the Strigolactone-Related Shoot Branching Pathway.” Current Opinion in Plant Biology 13 (1): 34–39.

Blake, Sara N., Karen M. Barry, Warwick M. Gill, James B. Reid, and Eloise Foo. 2016. “The Role of Strigolactones and Ethylene in Disease Caused by Pythium Irregulare.” Molecular Plant Pathology 17 (5): 680–90.

Bulgarelli, Davide, Ruben Garrido-Oter, Philipp C. Münch, Aaron Weiman, Johannes Dröge, Yao Pan, Alice C. McHardy, and Paul Schulze-Lefert. 2015. “Structure and Function of the Bacterial Root Microbiota in Wild and Domesticated Barley.” Cell Host & Microbe 17 (3): 392–403.

Bulgarelli, Davide, Klaus Schlaeppi, Stijn Spaepen, Emiel Ver Loren van Themaat, and Paul Schulze-Lefert. 2013. “Structure and Functions of the Bacterial Microbiota of Plants.” Annual Review of Plant Biology 64 (January): 807–38.

Caravaca, F., M. M. Alguacil, R. Azcón, and A. Roldán. 2006. “Formation of Stable

Aggregates in Rhizosphere Soil of Juniperus Oxycedrus: Effect of AM Fungi and Organic Amendments." Applied Soil Ecology 33 (1): 30–38.

Carvalhais, Lilia C., Vivian A. Rincon-Florez, Philip B. Brewer, Christine A. Beveridge, Paul G. Dennis, and Peer M. Schenk. 2019. "The Ability of Plants to Produce Strigolactones Affects Rhizosphere Community Composition of Fungi but Not Bacteria." Rhizosphere 9 (March): 18–26.

Castrillo, Gabriel, Paulo José Pereira Lima Teixeira, Sur Herrera Paredes, Theresa F. Law, Laura de Lorenzo, Meghan E. Feltcher, Omri M. Finkel, et al. 2017. "Root Microbiota Drive Direct Integration of Phosphate Stress and Immunity." Nature 543 (7646): 513–18.

Cheng, Xi, Carolien Ruyter-Spira, and Harro Bouwmeester. 2013. "The Interaction between Strigolactones and Other Plant Hormones in the Regulation of Plant Development." Frontiers in Plant Science 4 (June): 199.

Coleman-Derr, Devin, Damaris Desgarennes, Citlali Fonseca-Garcia, Stephen Gross, Scott Clingenpeel, Tanja Woyke, Gretchen North, Axel Visel, Laila P. Partida-Martinez, and Susannah G. Tringe. 2016. "Plant Compartment and Biogeography Affect Microbiome Composition in Cultivated and Native Agave Species." The New Phytologist 209 (2): 798–811.

Cook, C. E., L. P. Whichard, B. Turner, M. E. Wall, and G. H. Egley. 1966. "Germination of Witchweed (Striga Lutea Lour.): Isolation and Properties of a Potent Stimulant." Science 154 (3753): 1189–90.

Cregger, M. A., A. M. Veach, Z. K. Yang, M. J. Crouch, R. Vilgalys, G. A. Tuskan, and C. W. Schadt. 2018. “The Populus Holobiont: Dissecting the Effects of Plant Niches and Genotype on the Microbiome.” Microbiome 6 (1): 31.

Csardi, Gabor, and Tamas Nepusz. 2006. “The Igraph Software Package for Complex Network Research.” InterJournal, Complex Systems 1695 (5): 1–9.

De Cuyper, Carolien, Justine Fromentin, Rosita Endah Yocgo, Annick De Keyser, Bruno Guillotin, Karl Kunert, François-Didier Boyer, and Sofie Goormachtig. 2015. “From Lateral Root Density to Nodule Number, the Strigolactone Analogue GR24 Shapes the Root Architecture of Medicago Truncatula.” Journal of Experimental Botany 66 (13): 4091.

Deutscher, J., J. Deutscher, C. Francke, and P. W. Postma. 2008. “How Phosphotransferase System-Related Protein Phosphorylation Regulates Carbohydrate Metabolism in Bacteria.” Microbiology and Molecular Biology Reviews 70 (4): 939–1031.

Dor, Evgenia, Daniel M. Joel, Yoram Kapulnik, Hinanit Koltai, and Joseph Hershenhorn. 2011. “The Synthetic Strigolactone GR24 Influences the Growth Pattern of Phytopathogenic Fungi.” Planta 234 (2): 419–27.

Dor, Evgenia, Koichi Yoneyama, Smadar Wininger, Yoram Kapulnik, Kaori Yoneyama, Hinanit Koltai, Xiaonan Xie, and Joseph Hershenhorn. 2011. “Strigolactone Deficiency Confers Resistance in Tomato Line SL-ORT1 to the Parasitic Weeds Phelipanche and Orobanche Spp.” Phytopathology 101 (2): 213–22.

Edgar, Robert C., Brian J. Haas, Jose C. Clemente, Christopher Quince, and Rob Knight. 2011. “UCHIME Improves Sensitivity and Speed of Chimera Detection.” Bioinformatics 27 (16): 2194–2200.

Eilers, Kathryn G., Christian L. Lauber, Rob Knight, and Noah Fierer. 2010. “Shifts in Bacterial Community Structure Associated with Inputs of Low Molecular Weight Carbon Compounds to Soil.” Soil Biology & Biochemistry 42 (6): 896–903.

Fierer, Noah, Mark A. Bradford, and Robert B. Jackson. 2007. “Toward an Ecological Classification of Soil Bacteria.” Ecology 88 (6): 1354–64.

Flematti, Gavin R., Emilio L. Ghisalberti, Kingsley W. Dixon, and Robert D. Trengove. 2009. “Identification of Alkyl Substituted 2H-Furo[2,3-C]pyran-2-Ones as Germination Stimulants Present in Smoke.” Journal of Agricultural and Food Chemistry 57 (20): 9475–80.

Foo, Eloise, Sara N. Blake, Brendan J. Fisher, Jason A. Smith, and James B. Reid. 2016. “The Role of Strigolactones during Plant Interactions with the Pathogenic Fungus Fusarium Oxysporum.” Planta 243 (6): 1387–96.

Foo, Eloise, and Noel W. Davies. 2011. “Strigolactones Promote Nodulation in Pea.” Planta 234 (5): 1073–81.

Foo, Eloise, Kaori Yoneyama, Cassandra J. Hugill, Laura J. Quittenden, and James B. Reid. 2013. “Strigolactones and the Regulation of Pea Symbioses in Response to Nitrate and Phosphate Deficiency.” Molecular Plant 6 (1): 76–87.

Friedman, Jonathan, and Eric J. Alm. 2012. “Inferring Correlation Networks from Genomic Survey Data.” PLoS Computational Biology 8 (9): e1002687.

Goh, Chooi-Hua, Debora F. Veliz Vallejos, Adrienne B. Nicotra, and Ulrike Mathesius. 2013. “The Impact of Beneficial Plant-Associated Microbes on Plant Phenotypic Plasticity.” Journal of Chemical Ecology 39 (7): 826–39.

Goodstein, David M., Shengqiang Shu, Russell Howson, Rochak Neupane, Richard D. Hayes, Joni Fazo, Therese Mitros, et al. 2012. “Phytozome: A Comparative Platform for Green Plant Genomics.” Nucleic Acids Research 40 (Database issue): D1178–86.

Graham, T. L. 1991. “Flavonoid and Isoflavonoid Distribution in Developing Soybean Seedling Tissues and in Seed and Root Exudates.” Plant Physiology 95 (2): 594–603.

Grant, David, Rex T. Nelson, Steven B. Cannon, and Randy C. Shoemaker. 2010. “SoyBase, the USDA-ARS Soybean Genetics and Genomics Database.” Nucleic Acids Research 38 (Database issue): D843–46.

Han, Li-Li, Jun-Tao Wang, Sheng-Hui Yang, Wen-Feng Chen, Li-Mei Zhang, and Ji-Zheng He. 2017. “Temporal Dynamics of Fungal Communities in Soybean Rhizosphere.” Journal of Soils and Sediments 17 (2): 491–98.

Hassani, M. Amine, Paloma Durán, and Stéphane Hacquard. 2018. “Microbial Interactions within the Plant Holobiont.” Microbiome 6 (1): 58.

Ho, Adrian, D. Paolo Di Lonardo, and Paul L. E. Bodelier. 2017. "Revisiting Life Strategy Concepts in Environmental Microbial Ecology." FEMS Microbiology Ecology 93 (3): fix006.

Hodge, Angela. 2001. "Arbuscular Mycorrhizal Fungi Influence Decomposition Of, but Not Plant Nutrient Capture From, Glycine Patches in Soil." The New Phytologist 151 (3): 725–34.

Jones, Fred Reuel. 1924. "A Mycorrhizal Fungus in the Roots of Legumes and Some Other Plants." Journal of Agricultural Research 29 (9): 459–70.

Kereszt, Attila, Dongxue Li, Arief Indrasumunar, Cuc D. T. Nguyen, Sureeporn Nontachaiyapoom, Mark Kinkema, and Peter M. Gresshoff. 2007. "Agrobacterium Rhizogenes-Mediated Transformation of Soybean to Study Root Biology." Nature Protocols 2 (4): 948–52.

Kgosi, R. L., B. Zwanenburg, A. S. Mwakaboko, and A. J. Murdoch. 2012. "Strigolactone Analogues Induce Suicidal Seed Germination of Striga Spp. in Soil." Weed Research 52 (3): 197–203.

Khan, M. M. A., and W. D. Bauer. 1988. "Chemotaxis of Bradyrhizobium Japonicum towards Flavones and Isoflavones from Soybean (abstract No. 760)." Plant Physiology 86.

Kolde, Raivo. 2015. "Pheatmap: Pretty Heatmaps. R Package Version 1.0. 8."

Kozich, James J., Sarah L. Westcott, Nielson T. Baxter, Sarah K. Highlander, and

Patrick D. Schloss. 2013. “Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform.” Applied and Environmental Microbiology 79 (17): 5112–20.

Kretzschmar, Tobias, Wouter Kohlen, Joelle Sasse, Lorenzo Borghi, Markus Schlegel, Julien B. Bachelier, Didier Reinhardt, Ralph Bours, Harro J. Bouwmeester, and Enrico Martinoia. 2012. “A Petunia ABC Protein Controls Strigolactone-Dependent Symbiotic Signalling and Branching.” Nature 483 (7389): 341–44.

Lebeis, Sarah L., Sur Herrera Paredes, Derek S. Lundberg, Natalie Breakfield, Jase Gehring, Meredith McDonald, Stephanie Malfatti, et al. 2015. “Salicylic Acid Modulates Colonization of the Root Microbiome by Specific Bacterial Taxa.” Science 349 (6250): 860–64.

Lee, Eun-Hwa, Ju-Kyeong Eo, Kang-Hyeon Ka, and Ahn-Heum Eom. 2013. “Diversity of Arbuscular Mycorrhizal Fungi and Their Roles in Ecosystems.” Mycobiology 41 (3): 121–25.

Levy, Asaf, Isai Salas Gonzalez, Maximilian Mittelviefhaus, Scott Clingenpeel, Sur Herrera Paredes, Jiamin Miao, Kunru Wang, et al. 2018. “Genomic Features of Bacterial Adaptation to Plants.” Nature Genetics 50 (1): 138–50.

Lin, Dong Xu, En Tao Wang, Hui Tang, Tian Xu Han, Yu Rong He, Su Hua Guan, and Wen Xin Chen. 2008. “Shinella Kummerowiae Sp. Nov., a Symbiotic Bacterium Isolated from Root Nodules of the Herbal Legume Kummerowia Stipulacea.” International Journal of Systematic and Evolutionary Microbiology 58 (6): 1409–13.

Lindström, Kristina, Mazvita Murwira, Anne Willems, and Nora Altier. 2010. “The Biodiversity of Beneficial Microbe-Host Mutualism: The Case of Rhizobia.” Research in Microbiology 161 (6): 453–63.

Liu, Fang, Tarek Hewezi, Sarah L. Lebeis, Vince Pantalone, Parwinder S. Grewal, and Margaret E. Staton. 2019. “Soil Indigenous Microbiome and Plant Genotypes Cooperatively Modify Soybean Rhizosphere Microbiome Assembly.” BMC Microbiology 19 (1): 201.

López-Ráez, Juan A. 2016. “How Drought and Salinity Affect Arbuscular Mycorrhizal Symbiosis and Strigolactone Biosynthesis?” Planta 243 (6): 1375–85.

López-Ráez, Juan A., Ken Shirasu, and Eloise Foo. 2017. “Strigolactones in Plant Interactions with Beneficial and Detrimental Organisms: The Yin and Yang.” Trends in Plant Science 22 (6): 527–37.

Love, Michael I., Wolfgang Huber, and Simon Anders. 2014. “Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2.” Genome Biology 15 (12): 550.

Lugtenberg, Ben, and Faina Kamilova. 2009. “Plant-Growth-Promoting Rhizobacteria.” Annual Review of Microbiology 63: 541–56.

Lundberg, Derek S., Sarah L. Lebeis, Sur Herrera Paredes, Scott Yourstone, Jase Gehring, Stephanie Malfatti, Julien Tremblay, et al. 2012. “Defining the Core Arabidopsis Thaliana Root Microbiome.” Nature 488 (7409): 86–90.

Lundberg, Derek S., Scott Yourstone, Piotr Mieczkowski, Corbin D. Jones, and Jeffery L. Dangl. 2013. “Practical Innovations for High-Throughput Amplicon Sequencing.” Nature Methods 10 (10): 999–1002.

Lu, Tao, Mingjing Ke, W. J. G. M. Peijnenburg, Youchao Zhu, Meng Zhang, Liwei Sun, Zhengwei Fu, and Haifeng Qian. 2018. “Investigation of Rhizospheric Microbial Communities in Wheat, Barley, and Two Rice Varieties at the Seedling Stage.” Journal of Agricultural and Food Chemistry 66 (11): 2645–53.

Marschner, Horst, and Petra Marschner. 1995. “Mineral Nutrition of Higher Plants”. Gulf Professional Publishing.

Martínez-Hidalgo, Pilar, and Ann M. Hirsch. 2017. “The Nodule Microbiome: N2-Fixing Rhizobia Do Not Live Alone.” Phytobiomes Journal 1 (2): 70–82.

McAdam, Erin L., Cassandra Hugill, Sebastien Fort, Eric Samain, Sylvain Cottaz, Noel W. Davies, James B. Reid, and Eloise Foo. 2017. “Determining the Site of Action of Strigolactones during Nodulation.” Plant Physiology 175 (1): 529–42.

Mendes, Lucas W., Eiko E. Kuramae, Acácio A. Navarrete, Johannes A. van Veen, and Siu M. Tsai. 2014. “Taxonomical and Functional Microbial Community Selection in Soybean Rhizosphere.” The ISME Journal 8 (8): 1577–87.

Moscatiello, Roberto, Andrea Squartini, Paola Mariani, and Lorella Navazio. 2010. “Flavonoid-Induced Calcium Signalling in Rhizobium Leguminosarum Bv. Viciae.” The New Phytologist 188 (3): 814–23.

Mu, Yang, Wei-Bin Jia, Zhuang Ke, Wen Zhuang, Hong-Mei Wang, Jian-Dong Jiang, Kai Chen, and Qing Chen. 2018. “Shinella Pollutisoli Sp. Nov., Isolated from Tetrabromobisphenol A-Contaminated Soil.” International Journal of Systematic and Evolutionary Microbiology 68 (8): 2602–6.

Nelson, David C., Adrian Scaffidi, Elizabeth A. Dun, Mark T. Waters, Gavin R. Flematti, Kingsley W. Dixon, Christine A. Beveridge, Emilio L. Ghisalberti, and Steven M. Smith. 2011. “F-Box Protein MAX2 Has Dual Roles in Karrikin and Strigolactone Signaling in Arabidopsis Thaliana.” Proceedings of the National Academy of Sciences of the United States of America 108 (21): 8897–8902.

Neumann, Silke, Karin Grosse, and Victor Sourjik. 2012. “Chemotactic Signaling via Carbohydrate Phosphotransferase Systems in Escherichia Coli.” Proceedings of the National Academy of Sciences of the United States of America 109 (30): 12159–64.

Nguyen, Nhu H., Zewei Song, Scott T. Bates, Sara Branco, Leho Tedersoo, Jon Menke, Jonathan S. Schilling, and Peter G. Kennedy. 2016. “FUNGuild: An Open Annotation Tool for Parsing Fungal Community Datasets by Ecological Guild.” Fungal Ecology 20 (April): 241–48.

Nilsson, Rolf Henrik, Karl-Henrik Larsson, Andy F. S. Taylor, Johan Bengtsson-Palme, Thomas S. Jeppesen, Dmitry Schigel, Peter Kennedy, et al. 2019. “The UNITE Database for Molecular Identification of Fungi: Handling Dark Taxa and Parallel Taxonomic Classifications.” Nucleic Acids Research 47 (D1): D259–64.

Olanya, O. M., and D. K. Lakshman. 2015. “Potential of Predatory Bacteria as

Biocontrol Agents for Foodborne and Plant Pathogens.” Journal of Plant Pathology: An International Journal of the Italian Phytopathological Society 97 (3).

Peláez-Vico, María A., Lydia Bernabéu-Roda, Wouter Kohlen, María J. Soto, and Juan A. López-Ráez. 2016. “Strigolactones in the Rhizobium-Legume Symbiosis: Stimulatory Effect on Bacterial Surface Motility and down-Regulation of Their Levels in Nodulated Plants.” Plant Science: An International Journal of Experimental Plant Biology 245 (April): 119–27.

Pérez-Jaramillo, Juan E., Rodrigo Mendes, and Jos M. Raaijmakers. 2016. “Impact of Plant Domestication on Rhizosphere Microbiome Assembly and Functions.” Plant Molecular Biology 90 (6): 635–44.

Philippot, Laurent, Jos M. Raaijmakers, Philippe Lemanceau, and Wim H. van der Putten. 2013. “Going back to the Roots: The Microbial Ecology of the Rhizosphere.” Nature Reviews. Microbiology 11 (11): 789–99.

Pieterse, Corné M. J., Ronnie de Jonge, and Roeland L. Berendsen. 2016. “The Soil-Borne Supremacy.” Trends in Plant Science 21 (3): 171–73.

Promputtha, Itthayakorn, Saisamorn Lumyong, Vijaykrishna Dhanasekaran, Eric Huge Charles McKenzie, Kevin David Hyde, and Rajesh Jeewon. 2007. “A Phylogenetic Evaluation of Whether Endophytes Become Saprotrophs at Host Senescence.” Microbial Ecology 53 (4): 579–90.

Rognes, Torbjørn, Tomáš Flouri, Ben Nichols, Christopher Quince, and Frédéric Mahé.

2016. “VSEARCH: A Versatile Open Source Tool for Metagenomics.” PeerJ 4 (October): e2584.

Roy, K. W. 1997. “Fusarium Solani on Soybean Roots: Nomenclature of the Causal Agent of Sudden Death Syndrome and Identity and Relevance of F. Solani Form B.” Plant Disease 81 (3): 259–66.

Ruiz-Lozano, Juan Manuel, Ricardo Aroca, Ángel María Zamarreño, Sonia Molina, Beatriz Andreo-Jiménez, Rosa Porcel, José María García-Mina, Carolien Ruyter-Spira, and Juan Antonio López-Ráez. 2016. “Arbuscular Mycorrhizal Symbiosis Induces Strigolactone Biosynthesis under Drought and Improves Drought Tolerance in Lettuce and Tomato.” Plant, Cell & Environment 39 (2): 441–52.

Ruyter-Spira, Carolien, Salim Al-Babili, Sander van der Krol, and Harro Bouwmeester. 2013. “The Biology of Strigolactones.” Trends in Plant Science 18 (2): 72–83.

Scherff, R. H. 1973. “Control of Bacterial Blight of Soybean by Bdellovibrio Bacteriovorus.” Phytopathology 328: 400–02.

Schloss, Patrick D., Sarah L. Westcott, Thomas Ryabin, Justine R. Hall, Martin Hartmann, Emily B. Hollister, Ryan A. Lesniewski, et al. 2009. “Introducing Mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities.” Applied and Environmental Microbiology 75 (23): 7537–41.

Segata, Nicola, Jacques Izard, Levi Waldron, Dirk Gevers, Larisa Miropolsky, Wendy S.

Garrett, and Curtis Huttenhower. 2011. “Metagenomic Biomarker Discovery and Explanation.” Genome Biology 12 (6): R60.

Shi, Shengjing, Erin E. Nuccio, Zhou J. Shi, Zhili He, Jizhong Zhou, and Mary K. Firestone. 2016. “The Interconnected Rhizosphere: High Network Complexity Dominates Rhizosphere Assemblages.” Ecology Letters 19 (8): 926–36.

Shizuka, Daizaburo, and Damien R. Farine. 2016. “Measuring the Robustness of Network Community Structure Using Assortativity.” Animal Behaviour 112 (February): 237–46.

Singer, Esther, Jason Bonnette, Shawn C. Kenaley, Tanja Woyke, and Thomas E. Juenger. 2019. “Plant Compartment and Genetic Variation Drive Microbiome Composition in Switchgrass Roots.” Environmental Microbiology Reports 11 (2): 185–95.

Smith, Sally E., and David J. Read. 2010. Mycorrhizal Symbiosis. Academic Press.

Smith, Steven M., and Jiayang Li. 2014. “Signalling and Responses to Strigolactones and Karrikins.” Current Opinion in Plant Biology 21 (October): 23–29.

Soto, María J., Mónica Fernández-Aparicio, Vilma Castellanos-Morales, José M. García-Garrido, Juan A. Ocampo, María J. Delgado, and Horst Vierheilig. 2010. “First Indications for the Involvement of Strigolactones on Nodule Formation in Alfalfa (Medicago Sativa).” Soil Biology & Biochemistry 42 (2): 383–85.

Stringlis, Ioannis A., Ke Yu, Kirstin Feussner, Ronnie de Jonge, Sietske Van Bentum, Marcel C. Van Verk, Roeland L. Berendsen, Peter A. H. M. Bakker, Ivo Feussner, and Corné M. J. Pieterse. 2018. “MYB72-Dependent Coumarin Exudation Shapes Root Microbiome Assembly to Promote Plant Health.” Proceedings of the National Academy of Sciences of the United States of America 115 (22): E5213–22.

Takahashi, Shunsuke, Junko Tomita, Kaori Nishioka, Takayoshi Hisada, and Miyuki Nishijima. 2014. “Development of a Prokaryotic Universal Primer for Simultaneous Analysis of Bacteria and Archaea Using next-Generation Sequencing.” PloS One 9 (8): e105592.

Timm, C. M., K. R. Carter, A. A. Carrell, S. R. Jun, and S. S. Jawdy. 2018. “Abiotic Stresses Shift Belowground Populus-Associated Bacteria toward a Core Stress Microbiome.” MSystems 3 (1): 00070–17.

Torres-Vera, Rocío, Juan M. García, María J. Pozo, and Juan A. López-Ráez. 2014. “Do Strigolactones Contribute to Plant Defence?” Molecular Plant Pathology 15 (2): 211–16.

Untergasser, Andreas, Ioana Cutcutache, Triinu Koressaar, Jian Ye, Brant C. Faircloth, Maido Remm, and Steven G. Rozen. 2012. “Primer3—new Capabilities and Interfaces.” Nucleic Acids Research 40 (15): e115-e115.

Urbina, Hector, Martin F. Breed, Weizhou Zhao, Kanaka Lakshmi Gurrala, Siv G. E. Andersson, Jon Ågren, Sandra Baldauf, and Anna Rosling. 2018. “Specificity in Arabidopsis Thaliana Recruitment of Root Fungal Communities from Soil and Rhizosphere.” Fungal Biology 122 (4): 231–40.

Van Ha, Chien, Marco Antonio Leyva-González, Yuriko Osakabe, Uyen Thi Tran, Rie Nishiyama, Yasuko Watanabe, Maho Tanaka, et al. 2014. “Positive Regulatory Role of Strigolactone in Plant Responses to Drought and Salt Stress.” Proceedings of the National Academy of Sciences of the United States of America 111 (2): 851–56.

Vaz-Moreira, Ivone, Cátia Faria, Ana R. Lopes, Liselott A. Svensson, Edward R. B. Moore, Olga C. Nunes, and Célia M. Manaia. 2010. “Shinella Fusca Sp. Nov., Isolated from Domestic Waste Compost.” International Journal of Systematic and Evolutionary Microbiology 60 (Pt 1): 144–48.

Visentin, Ivan, Marco Vitali, Manuela Ferrero, Yanxia Zhang, Carolien Ruyter-Spira, Ondřej Novák, Miroslav Strnad, Claudio Lovisolo, Andrea Schubert, and Francesca Cardinale. 2016. “Low Levels of Strigolactones in Roots as a Component of the Systemic Signal of Drought Stress in Tomato.” The New Phytologist 212 (4): 954–63.

Wang, Qiong, George M. Garrity, James M. Tiedje, and James R. Cole. 2007. “Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy.” Applied and Environmental Microbiology 73 (16): 5261–67.

Waters, Mark T., Caroline Gutjahr, Tom Bennett, and David C. Nelson. 2017. “Strigolactone Signaling and Evolution.” Annual Review of Plant Biology 68 (April): 291–322.

Weiss, Sophie, Will Van Treuren, Catherine Lozupone, Karoline Faust, Jonathan Friedman, Ye Deng, Li Charlie Xia, et al. 2016. “Correlation Detection Strategies in

Microbial Data Sets Vary Widely in Sensitivity and Precision.” The ISME Journal 10 (7): 1669–81.

Wei, Zhong, Tianjie Yang, Ville-Petri Friman, Yangchun Xu, Qirong Shen, and Alexandre Jousset. 2015. “Trophic Network Architecture of Root-Associated Bacterial Communities Determines Pathogen Invasion and Plant Health.” Nature Communications 6 (September): 8413.

Wemheuer, Franziska, Jessica A. Taylor, Rolf Daniel, Emma Johnston, Peter Meinicke, Torsten Thomas, and Bernd Wemheuer. 2018. “Tax4Fun2: A R-Based Tool for the Rapid Prediction of Habitat-Specific Functional Profiles and Functional Redundancy Based on 16S rRNA Gene Marker Gene Sequences.” bioRxiv: 490037.

Williams, Alwyn, Lokeshwaran Manoharan, Nicholas P. Rosenstock, Pål Axel Olsson, and Katarina Hedlund. 2017. “Long-Term Agricultural Fertilization Alters Arbuscular Mycorrhizal Fungal Community Composition and Barley (Hordeum Vulgare) Mycorrhizal Carbon and Phosphorus Exchange.” The New Phytologist 213 (2): 874–85.

Wrather, J. A., T. R. Anderson, D. M. Arsyad, Y. Tan, L. D. Ploper, A. Porta-Puglia, H. H. Ram, and J. T. Yorinori. 2001. “Soybean Disease Loss Estimates for the Top Ten Soybean-Producing Counries in 1998.” Canadian Journal of Plant Pathology 23 (2): 115–21.

Wright, S. F., and A. Upadhyaya. 1998. “A Survey of Soils for Aggregate Stability and Glomalin, a Glycoprotein Produced by Hyphae of Arbuscular Mycorrhizal Fungi.”

Plant and Soil 198 (1): 97–107.

Xie, Xiaonan, Kaori Yoneyama, and Koichi Yoneyama. 2010. “The Strigolactone Story.” Annual Review of Phytopathology 48: 93–117.

Xu, Ling, Dan Naylor, Zhaobin Dong, Tuesday Simmons, Grady Pierroz, Kim K. Hixson, Young-Mo Kim, et al. 2018. “Drought Delays Development of the Sorghum Root Microbiome and Enriches for Monoderm Bacteria.” Proceedings of the National Academy of Sciences of the United States of America 115 (18): E4284–93.

Yan, Yan, Eiko E. Kuramae, Mattias de Hollander, Peter G. L. Klinkhamer, and Johannes A. van Veen. 2017. “Functional Traits Dominate the Diversity-Related Selection of Bacterial Communities in the Rhizosphere.” The ISME Journal 11 (1): 56–66.

Yoneyama, Kaori, Xiaonan Xie, Dai Kusumoto, Hitoshi Sekimoto, Yukihiro Sugimoto, Yasutomo Takeuchi, and Koichi Yoneyama. 2007. “Nitrogen Deficiency as Well as Phosphorus Deficiency in Sorghum Promotes the Production and Exudation of 5-Deoxystrigol, the Host Recognition Signal for Arbuscular Mycorrhizal Fungi and Root Parasites.” Planta 227 (1): 125–32.

Yoneyama, Kaori, Xiaonan Xie, Hitoshi Sekimoto, Yasutomo Takeuchi, Shin Ogasawara, Kohki Akiyama, Hideo Hayashi, and Koichi Yoneyama. 2008. “Strigolactones, Host Recognition Signals for Root Parasitic Plants and Arbuscular Mycorrhizal Fungi, from Fabaceae Plants.” The New Phytologist 179 (2): 484–94.

Zhalnina, Kateryna, Katherine B. Louie, Zhao Hao, Nasim Mansoori, Ulisses Nunes da Rocha, Shengjing Shi, Heejung Cho, et al. 2018. “Dynamic Root Exudate Chemistry and Microbial Substrate Preferences Drive Patterns in Rhizosphere Microbial Community Assembly.” Nature Microbiology 3 (4): 470–80.

Zhang, Baogang, Jun Zhang, Yao Liu, Peng Shi, and Gehong Wei. 2018. “Co-Occurrence Patterns of Soybean Rhizosphere Microbiome at a Continental Scale.” Soil Biology & Biochemistry 118 (March): 178–86.

Appendices 3

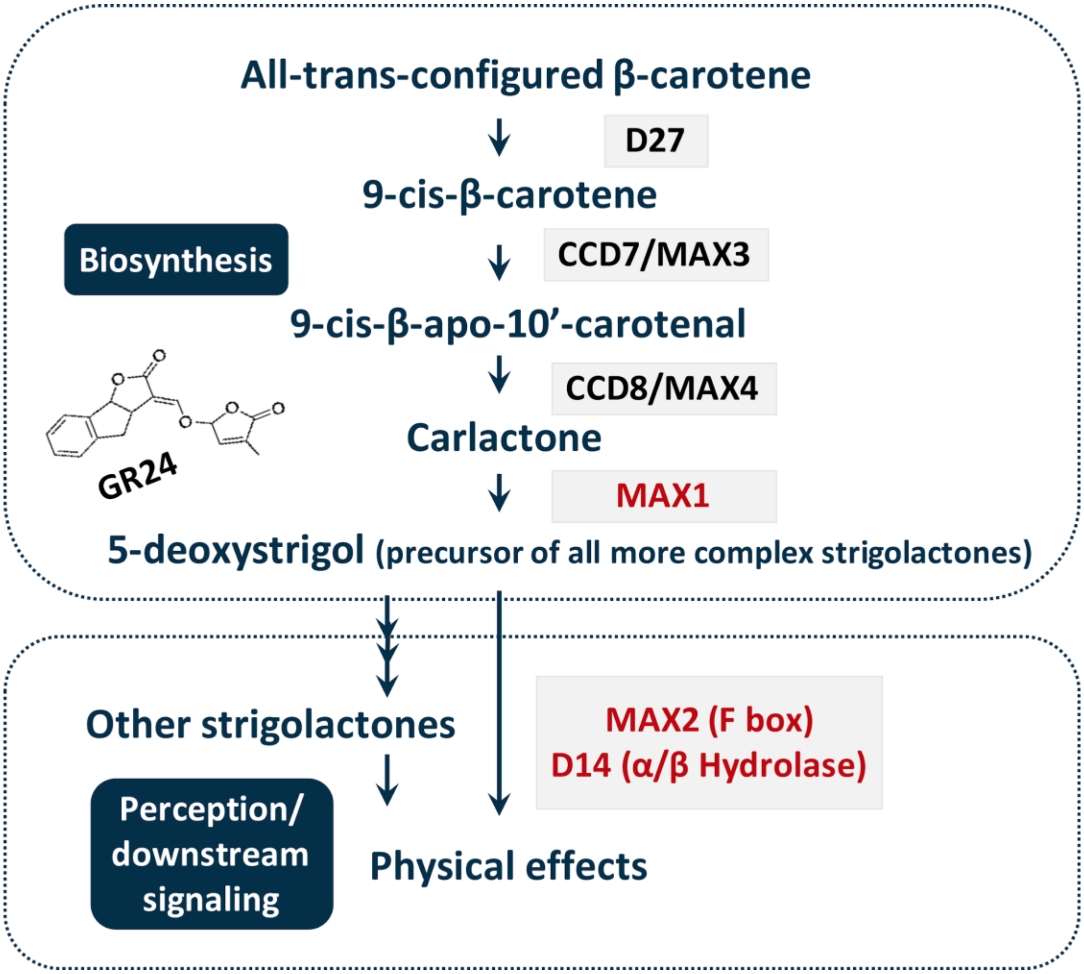


Fig. 3.1 Strigolactone signaling pathway and target genes for generating overexpression constructs.

Gene highlighted in red color, including Max1, Max2 and D14, are target genes for generating overexpression constructs.

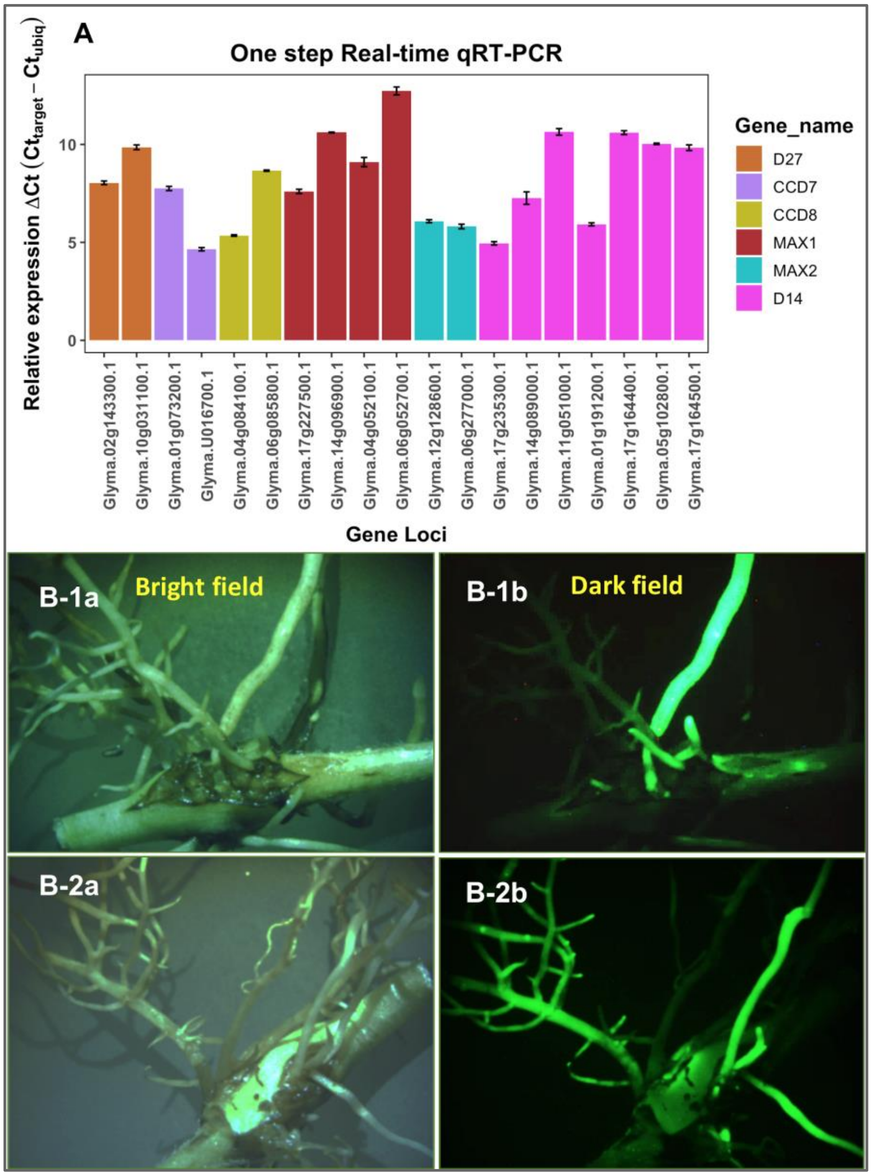


Fig. 3.2 Strigolactone candidate gene selection and overexpression confirmation.

(A) The relative expression across 19 homologs involved in strigolactone signaling pathway using ubiquitin as the housekeeping gene. Gene names listed matched with that illustrated in Fig 1; (B) Green fluorescent protein (GFP) expression in soybean transgenic hairy roots mediated by *Agrobacterium rhizogenes* K599. Figures B-1a and B-1b are the same root visualized under visable light and GFP specific filter respectively.

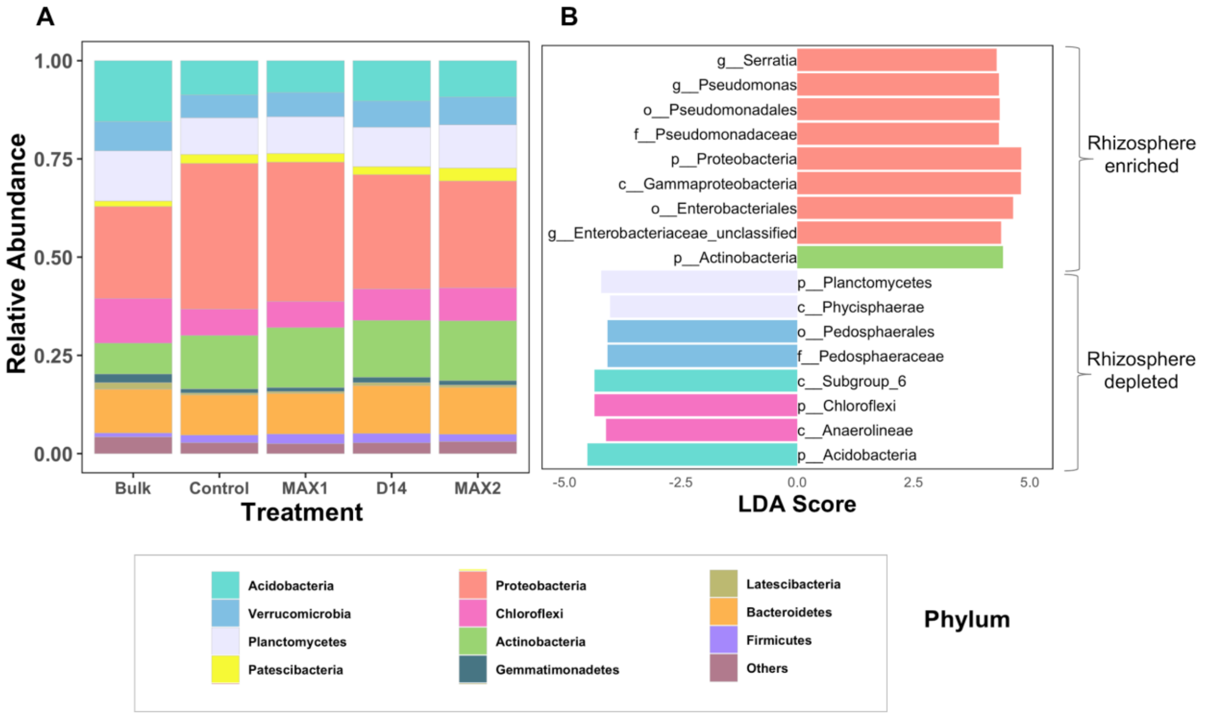


Fig. 3.3 The stacked barplot of bacteria phyla between treatments and differentially abundant analysis results between bulk soil and soybean rhizosphere community.

(A) The relative abundance of each bacterial phylum was calculated as the mean between replicates (7 or 8 reps). Different phyla were annotated as different colors; (B) Differential abundance analysis of individual bacteria taxa between rhizosphere and bulk soil. All bacteria taxa exhibited in the plot were all significantly enriched or depleted in the soybean rhizosphere. LDA score was used to indicate the effect size of each of the differentially abundant bacterial taxa. The larger this score, the more different of corresponding bacteria abundance between treatment.

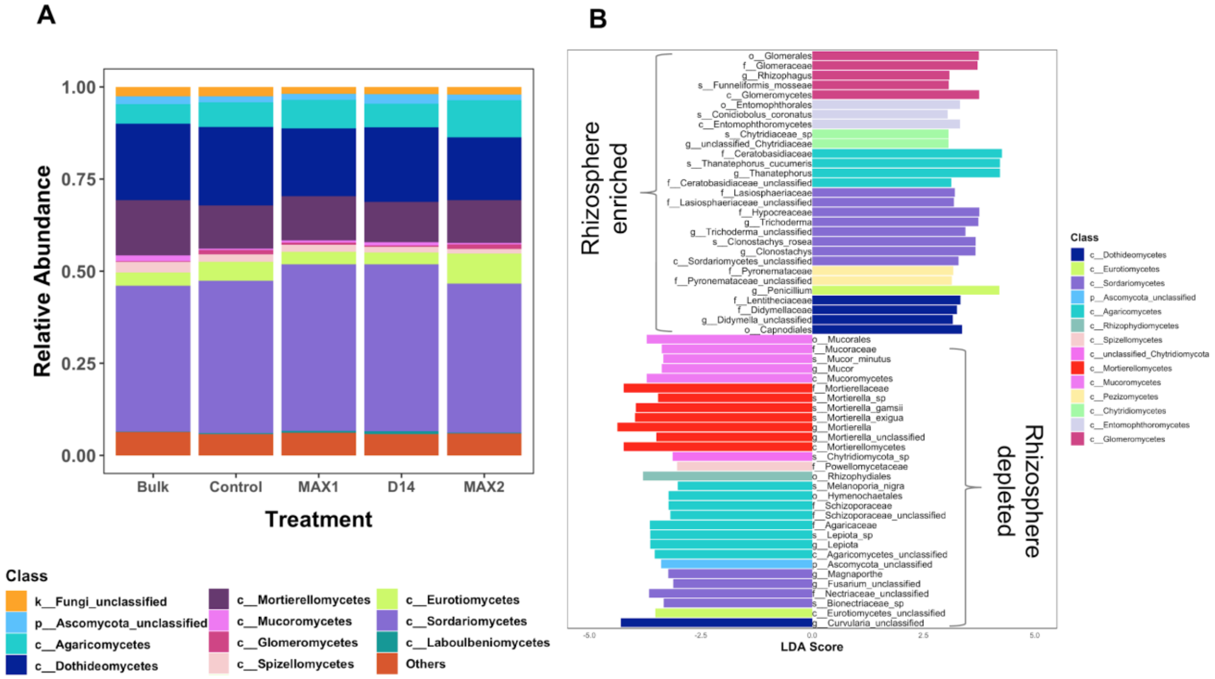


Fig. 3.4 Differential abundance analysis for bacteria and fungi taxa.

(A) Fungi composition at class level; (B) Differential abundance analysis of bacteria taxa between bulk soil and soybean rhizosphere community.

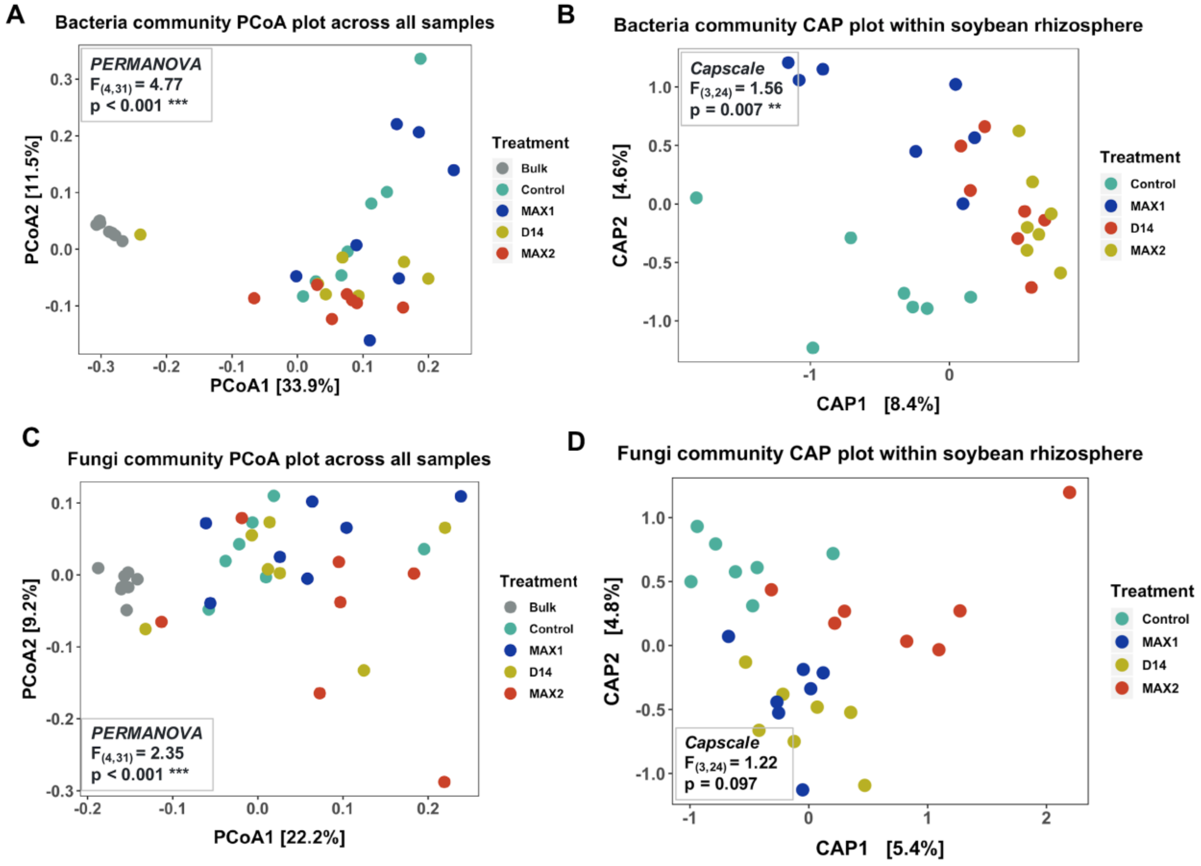


Fig. 3 5 Principle coordinate analysis (PCoA) and constrained analysis of principle coordinate analysis (CAP) plot for both bacteria and fungi community between treatments.

The permutational multivariate analysis of variance (PERMANOVA) and CAP test results were listed within each subplot.

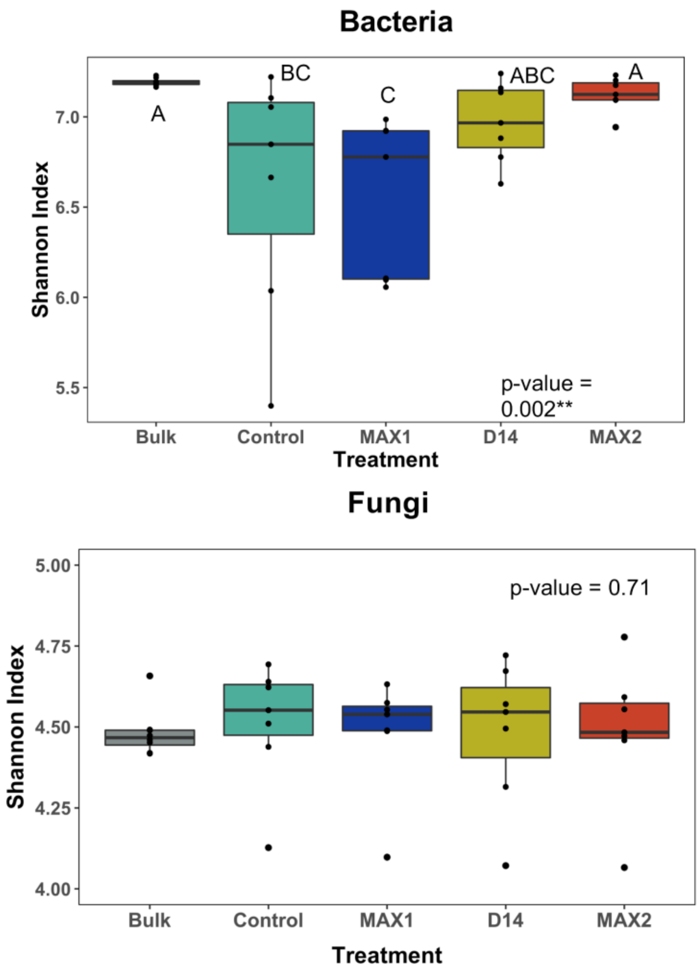


Fig. 3.6 Shannon diversity box dot plot for both bacteria and fungi community.

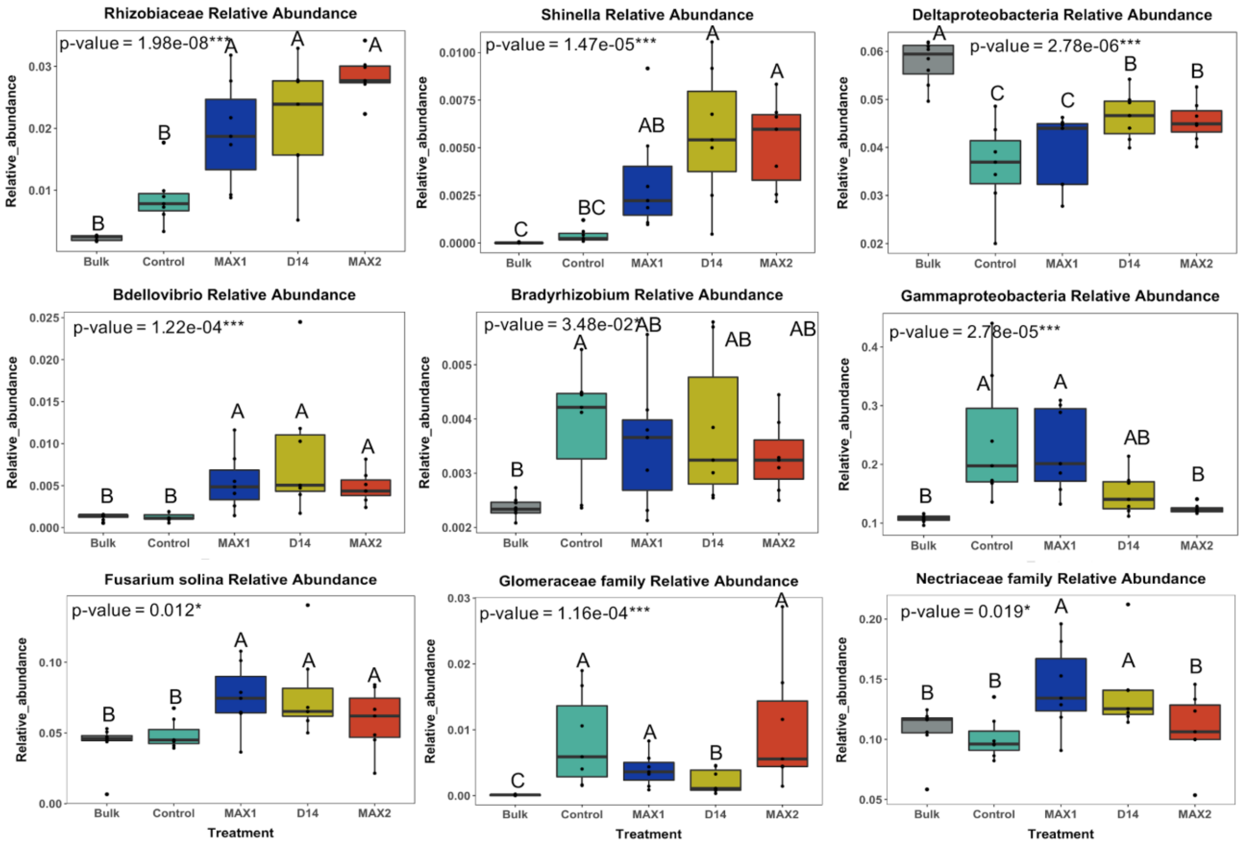


Fig. 3.7 Relative abundance barplot for individual bacterial and fungi taxa.

The p_value was calculated based either on one-way ANOVA or non-parametric Kruskal-wallis test depending on the normality of the data. The different characters on top of each bar represent the difference between treatments based on a pairwise comparison based either on TukeyHSD or Dunn’s Test.

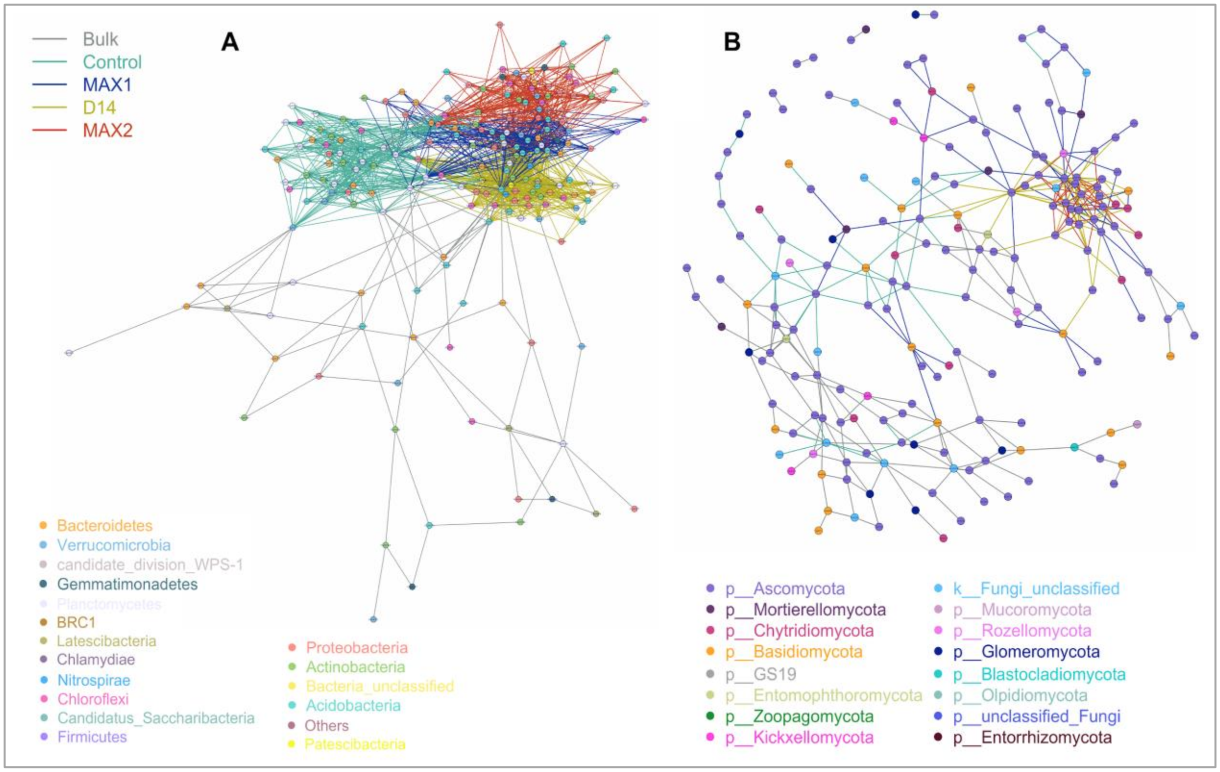


Fig. 3.8 Bacteria and fungi co-occurrence network calculated using SparCC algrithm and visualized in iGraph.

Different colors of the nodes represent different phylum (bacteria) or class (fungi) they belong to. Different colors of the edges represent different treatments as illustrated in the legend.

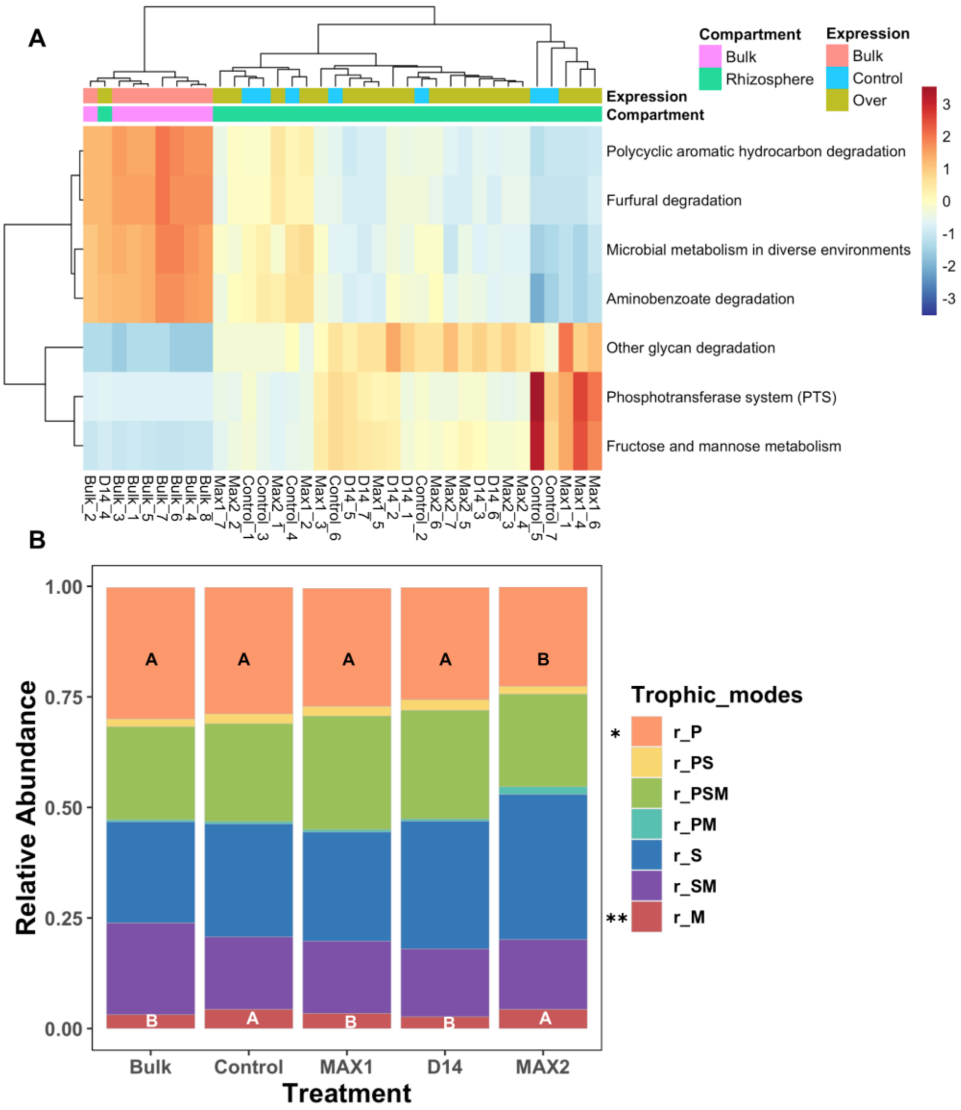


Fig. 3.9 Predicted function profiles for both bacterial and fungal communities.

(A) Bacteria metabolic pathway heatmap based on Tax4Fun2 functional annotation; (B) Stacked barplot of fungi trophic modes based on FUNGuild mediated functional annotation.

Table 3.1 Genes participating in strigolactone biosynthesis and perception in Arabidopsis and Williams 82.

Role	Arabidopsis Loci	Gene name	Protein identity/function	Soybean Loci	qRT-PCR forward primer	qRT-PCR reverse primer
Strigolactone biosynthesis	At1g03055	D27	9-cis/all trans-β-Carotene isomerase	Glyma.02g143300	AGTAATTGGCCACGTTGTGC	GGTATCGGGTGTGTTACATGC
	At1g03055			Glyma.10g031100	TGACATCACAGGGTTTCAGG	TGCATTGGACCACTTAGCTG
	At2g44990	CCD7/Max3/Rms5	Carotenoid cleavage dioxygenase 7	Glyma.01g073200	TGATGTATGGGAGGTTGCTG	CTTCAGCATTGCAAGACACG
	At2g44990			Glyma.U016700	CCTTCACATGCCAACACAAC	TGAATGGGTTTGGCTTGC
	At4g32810	CCD8/Max4/Rms1	Carotenoid cleavage dioxygenase 8	Glyma.04g084100	CACTCTAAACGACAAGGGCTTC	GAAAGCCATGATCGAAGTGG
	At4g32810			Glyma.06g085800	TGGACTTCCATATGGGTTGC	TGCGTCGTTTCTCTAAGCAG
	At2g26170	Max1	Cytochrome P450	Glyma.04g052100	TCGTTCTTCGTGCCTTTCAC	ACCATGGATTGGTGCTGAAC
	At2g26170			Glyma.06g052700	AAGGCTTCAGCCTGAGATTG	CGAACACGTGCCTCATTTAC
	At2g26170			Glyma.17g227500	TGTTCACCACCCTTCTGATG	TAAGGGAAGGTGTCCTACCAG
	At2g26170			Glyma.14g096900	TGGTATGGTTCTCAACTTCAAGC	TCCATTGCATCACAGTATTACG
Strigolactone perception /signaling	At3g03990	D14/D88/DAD2	α/β-hydrolases superfamily protein	Glyma.17g235300	GTTGCTTGCTTGTGTTTCTCC	TTCAGTTGTCTGGCCTCATC
	At3g03990			Glyma.14g089000	AGATTGCGCTTTCCCAGTAG	TAGGACCCTGCCTATTCTTCTC
	At4g37470	KAI2		Glyma.05g102800	GCCTCGACATTGCAACTAGAC	CCACACCACACCATCAATC

Table 3.1 Continued.

Role	Arabidopsis Loci	Gene name	Protein identity/function	Soybean Loci	qRT-PCR forward primer	qRT-PCR reverse primer
Strigolactone perception/ signaling	At4g37470			Glyma.11g051000	GGTCATTTGCCGCAGTTAAG	CCACAAACACACACAGGGATAG
	At4g37470			Glyma.01g191200	TCGTTATTCCGGTGTTGCTC	CATCACGTCGATGTCTACCTTC
	At4g37470			Glyma.17g164500	TATTCGTCTGGTCCTTCTCTCC	CAATGAGATTGACGCGTAGC
	At4g37470			Glyma.17g164400	AGCGAACAAACCCAACAATC	TGTGAGCCTCTTCCACGATAC
	At2g26170	Max2/D3/Rms4	F-box protein	Glyma.12g128600	GGGTGATGGTGGTGAATTG	CTTGCCACAGAAACCATTGC
	At2g26170			Glyma.06g277000	TGTATGGACCCATCCAATCC	CCAAGGGAATGGGTCTAATG

Table 3.2 Network topological features of bacterial and fungal community.

Community	**Network type**	**Network topologies**	**Bulk**	**Control**	**MAX1**	**D14**	**MAX2**
Bacteria	**Top50**	Positive ratio[a] (%)	67.09	87.89	67.24	63.41	49.85
		Edge density[b] (%)	7.63	28.98	28.65	36.82	26.86
		Ave. degree[c] (centralization)	3.43 (0.08)	14.2 (0.40)	14.04 (0.26)	18.04 (0.26)	13.16 (0.24)
		Ave.betweenness[d](centralization)	61.41 (0.16)	18.86 (0.11)	23.52 (0.04)	17.4 (0.06)	20.2 (0.05)
		Ave.closeness[e] (centralization)	0.27 (0.12)	0.57 (0.40)	0.53 (0.28)	0.60 (0.26)	0.56 (0.24)
		Modularity[f]	0.64	0.25	0.21	0.15	0.13
	Global	Positive ratio[a] (%)	55.66	55.56	54.07	54.9	51.91
		Edge density[b] (%)	0.96	1.73	1.7	3.25	1.52
		Ave. degree[c] (centralization)	4.44 (0.03)	7.16 (0.18)	7.60 (0.07)	15.05 (0.20)	7.08 (0.06)
		Ave.betweenness[d](centralization)	819.80 (0.05)	544.34 (0.14)	742.78 (0.04)	505.13 (0.10)	695.56 (0.15)
		Ave.closeness[e] (centralization)	0.07 (0.01)	0.05 (0.01)	0.09 (0.02)	0.11 (0.03)	0.06 (0.01)
		Modularity[f]	0.59	0.36	0.43	0.37	0.45
Fungi	**Top50**	Positive ratio[a] (%)	81.95	72.22	63.16	67.65	51.06
		Edge density[b] (%)	14.06	5.98	5.51	6.87	5.73
		Ave. degree[c] (centralization)	6.04 (0.21)	2.51 (0.08)	2.48 (0.08)	3.02 (0.09)	2.29 (0.07)
		Ave.betweenness[d] (centralization)	22.75 (0.09)	90.28 (0.37)	24.24 (0.11)	79.44 (0.41)	10.78 (0.08)
		Ave.closeness[e] (centralization)	0.11 (0.04)	0.11 (0.08)	0.04 (0.02)	0.14 (0.10)	0.04 (0.02)
		Modularity[f]	0.17	0.66	0.61	0.6	0.6

Table 3.2 Continued.

Community	Network type	Network topologies	Bulk	Control	MAX1	D14	MAX2
Fungi	**Global**	Positive ratio[a] (%)	55.63	54.22	51.98	53.36	52.33
		Edge density[b] (%)	0.91	0.72	0.72	0.81	0.75
		Ave. degree[c] (centralization)	4.13 (0.03)	3.34 (0.04)	3.19 (0.03)	3.56 (0.04)	3.26 (0.02)
		Ave.betweenness[d](centralization)	918.11 (0.06)	1026.44 (0.11)	994.32 (0.09)	984.30 (0.14)	1046.98 (0.05)
		Ave.closeness[e] (centralization)	0.08 (0.02)	0.07 (0.03)	0.03 (0.007)	0.07 (0.02)	0.08 (0.02)
		Modularity[f]	0.57	0.66	0.68	0.65	0.66

[a] Positive ratio represents the ratio of positive microbe-microbe correlations out of all interactions within the network.

[b] Edge density was calculated as the ratio of detected edge numbers to the theoretical maximum edge numbers, indicating the connectiveness between nodes.

[c] Ave. degree was defined as the mean connection degree across all nodes within a network.

[d] Ave. betweenness was defined to quantify how many times a node acts as a bridge along the shortest path connection the other two nodes, averaged across all nodes.

[e] Ave.closeness was calculated as the reciprocal of the sum of all the shortest path lengths between one node and all the remaining nodes.

[f] Modularity: the strength of division of a network into modules.

Inside the parentheses is the centralization value, which is used to define to what extent there are a small number of highly centralized nodes. When centralization equals to zero, it indicates that all nodes are equally central.

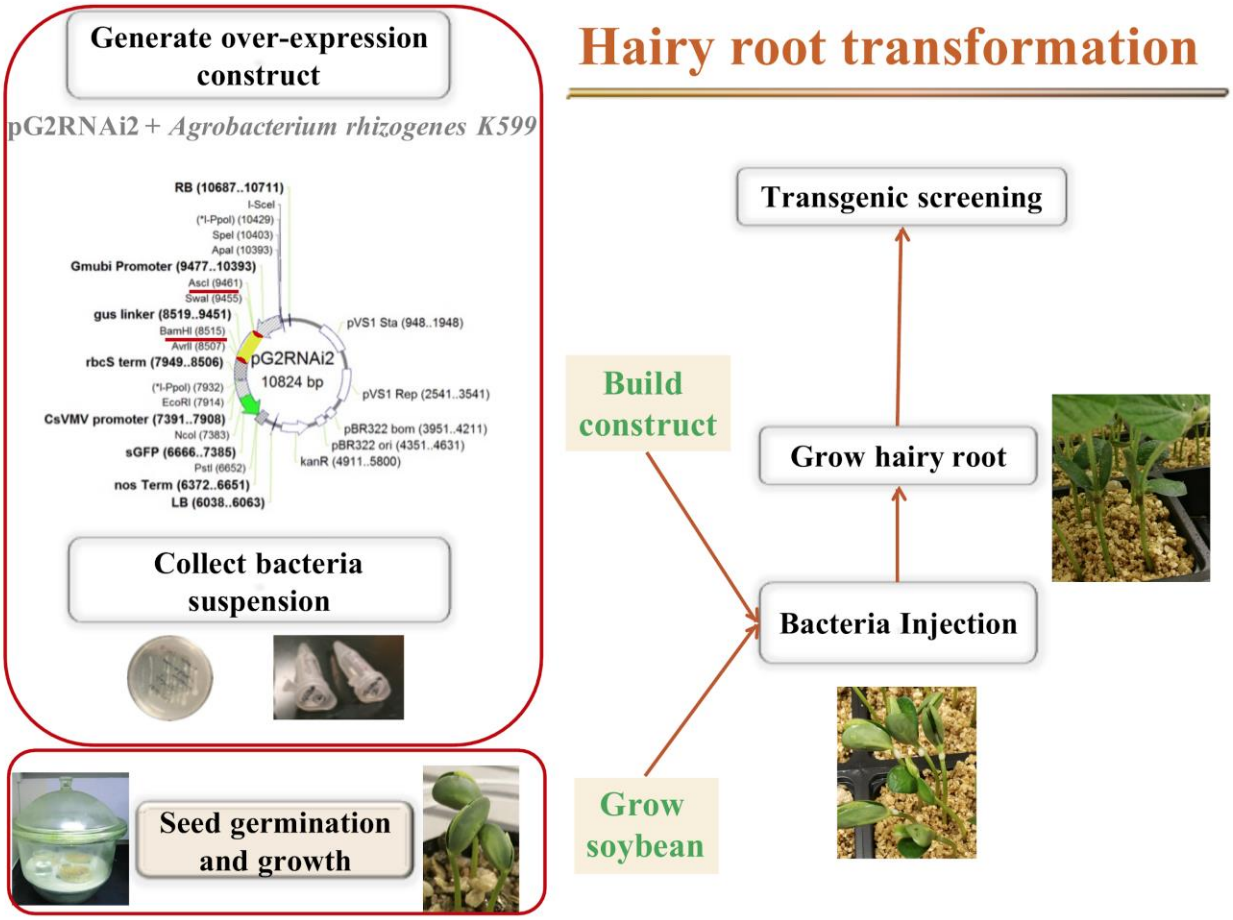


Fig. S3.1 Soybean hairy root transformation paradigm.

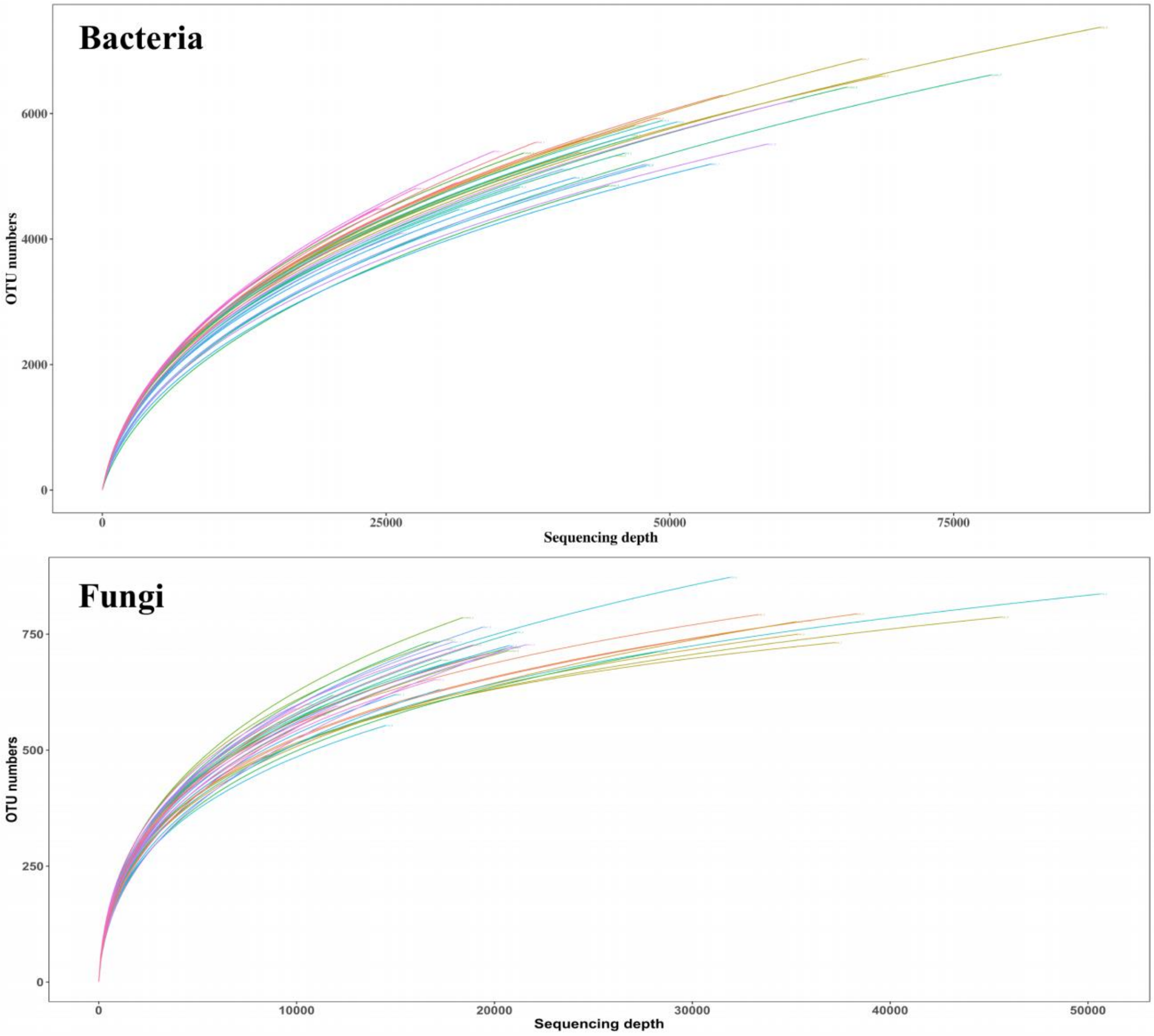


Fig. S3.2 Bacterial and fungal OTU rarefaction plot.

This plot demonstrated how OTU richness increased along with increased sequencing depth. When this plot reached the plateau, it indicated that the sequencing depth at this point is sufficient to represent the actual species richness.

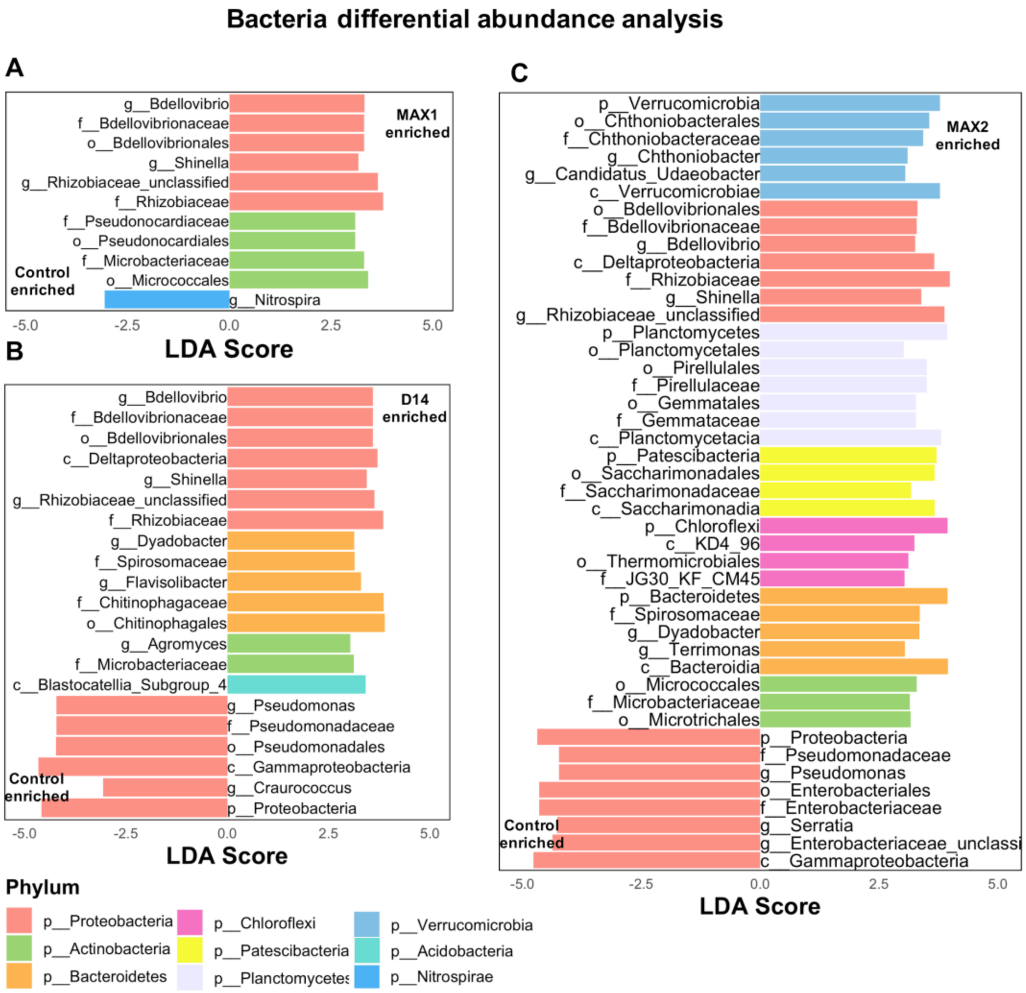


Fig. S3.3 Bacteria differential abundance analysis between overexpression constructs and control.

The differential abundance analysis was conducted using LefSe software across all taxonomic levels. All of the listed taxa were either significantly enriched or depleted under the overexpression of the specific genes. LDA score estimated the effect sized of each differentially abundant taxon.

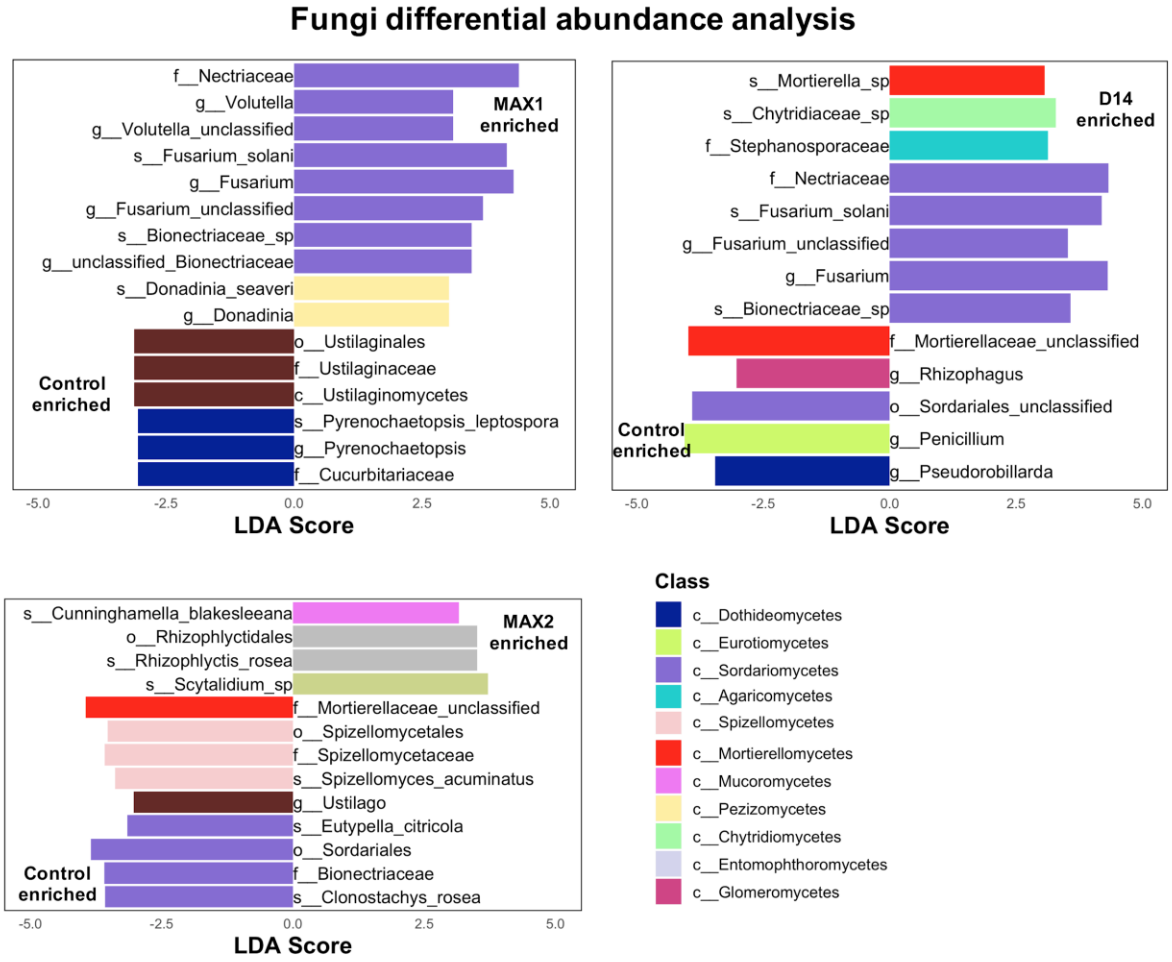


Fig. S3.4 Fungi differential abundance analysis between overexpression constructs and control.

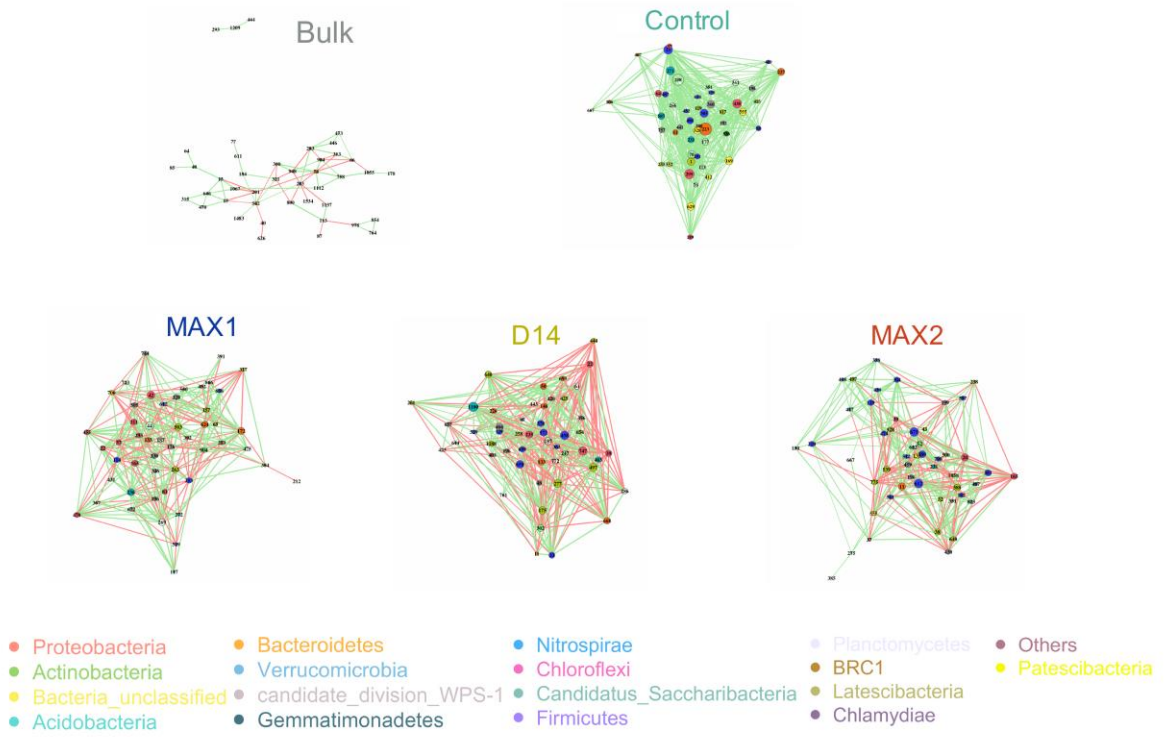


Fig. S3.5 Individual bacteria co-occurrence networks.

Each node represents one OTU, while each edge represents a significant correlation between two nodes. Different colors of the nodes represent different phyla each nodes belongs to as illustrated in the bottom of the figure. The color of the edges were assigned based on the correlations, with red and green color represents negative and positive correlations respectively.

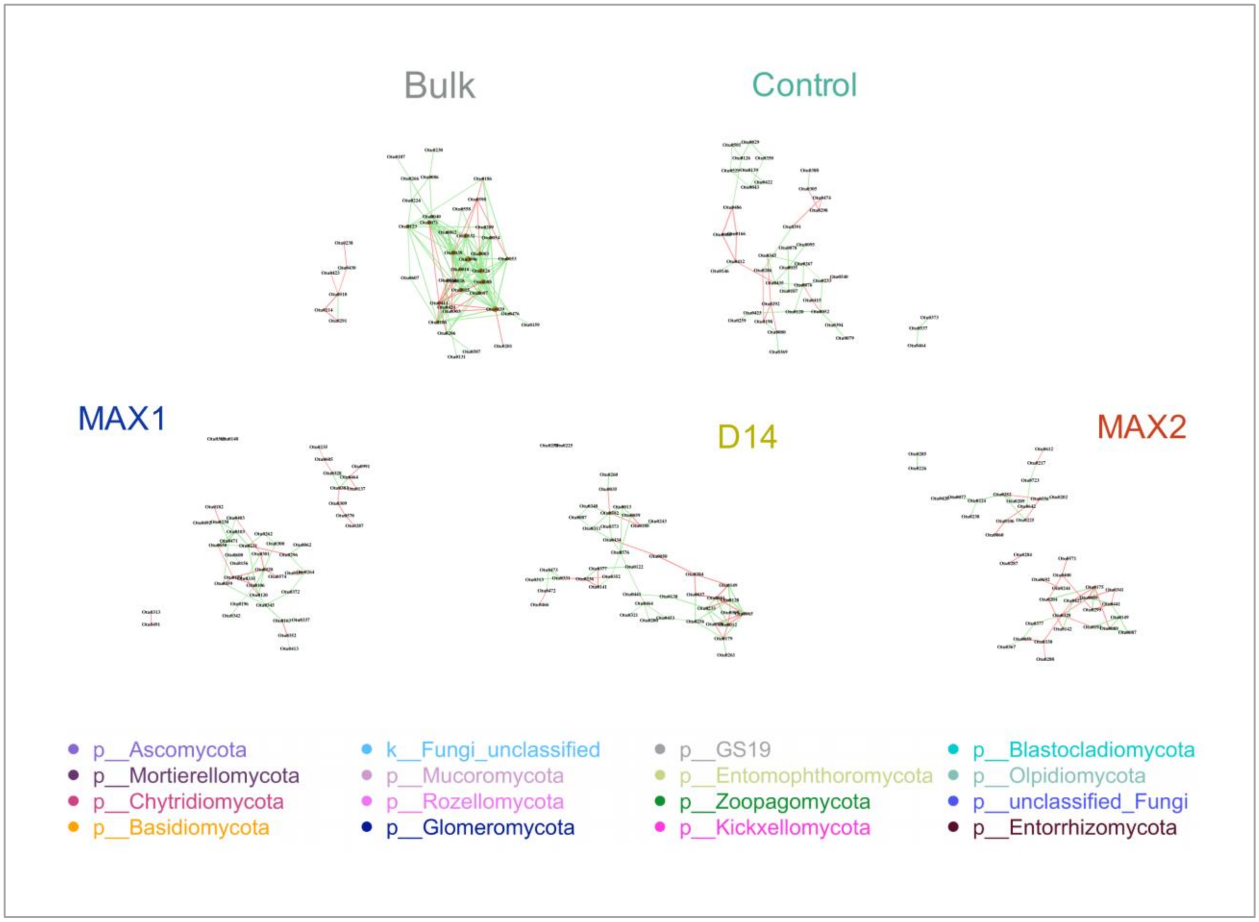


Fig. S3.6 Individual fungi co-occurrence networks.

Each node represents one OTU, while each edge represents a significant correlation between two nodes. Different colors of the nodes represent different phyla each node belongs to as illustrated in the bottom of the figure. The color of the edges was assigned based on the correlations, with red and green color represents negative and positive correlations respectively.

Table S3.1: Bacteria and fungi taxonomy and abundance (attached file).

Table S3.2: LefSe analysis results (attached file).

Table S3.3: Bacterial network-shared nodes and edges between treatments (attached file).

Table S3.4: Fungal network-shared nodes and edges between treatments (attached file).

File S3.1: Further investigation of enriched *Rhizobium* genus abundance in the rhizosphere of strigolactone overexpression constructs (attached file).

Chapter 4 Who is driving soybean root microbiome assembly and development - microbes from the seed or the soil indigenous community?

Declaration

The material presented in this chapter will be used for journal article publication.

Abstract

Plants have developed intimate relationships with microorganisms that promote better fitness and contribute to responses to changing biotic and abiotic growing conditions. While profiling of root-associated microbes is becoming more commonplace, more information is needed to understand the early stages of how these microbial communities are assembled and what factors may disrupt this assembly. In this study, we comprehensively characterized the dynamic assembly of the root endosphere and rhizosphere microbiome during the early development of soybean seedlings (1-4 weeks), emphasizing the relative contributions of seed-borne microbes versus soil indigenous microbes. Further, we explore the impact of fungicide seed treatment, a common agricultural practice, during this assembly process. Our results indicated that fungicide seed treatment impacts were insignificant for the bacterial microbiome, but caused a minor but significant decrease of the fungal genus *Chaetomium* in the soybean rhizosphere at week 3. We observed distinct bacterial and fungal community composition between bulk, rhizosphere and root endosphere compartments, which was detectable as early as one week after germination and with further changes in subsequent weeks. *Gammaproteobacteria* and *Actinobacteria* were dramatically decreased in the soybean rhizosphere and endosphere respectively one week after germination. The changes of fungal communities were less evident across early time points and showed higher between plot variation in comparison to bacteria. Dominant seed microbial taxa, including *Pantoea*, *Pseudomonas,* and *Methylobacterium*, strikingly decreased within the soybean root one week after germination. However, a few taxa originating from the seed remained after germination, such as *Bacillus* with a high

abundance within the root but not in the rhizosphere. Predicted bacterial metabolic pathways revealed distinct functional potentials between compartments and development stages. The bacterial functions involved in the inositol degradation and seed mucilage (D_galacturonate) degradation pathway were more abundant in the soybean rhizosphere at week one, while aromatic compound degradation related pathway were enriched for endosphere bacterial community, which greatly diversified along with soybean development. Fungicide seed treatment impacts were insignificant for the bacterial microbiome, but caused a minor but significant decrease of the fungal genus *Chaetomium* in the soybean rhizosphere at week 3.

4.1 Introduction

Plants have evolved intimate partnerships with surrounding microorganisms, a strategy enabling these sessile organisms to dynamically respond to changing biotic and abiotic environmental conditions and promote phenotypic/physiological plasticity (Beattie 2018; Vandenkoornhuyse et al. 2015; Bulgarelli et al. 2013; Müller et al. 2016). Members of the plant-associated microbiome can benefit plant health by improving nutrient availability/uptake, enhancing abiotic stress tolerance and promoting biotic defense (Goh et al. 2013; Schlaeppi and Bulgarelli 2015). Plant-associated microbial communities show a high degree of consistency in terms of phylogenetic structure across different plant species, which indicates some conserved mutual adaptation between plants and their microbial partners (Müller et al. 2016). Functional characterizations of these plant-associated microbiomes, based on community (metagenomic) sequencing and isolate genome sequencing, have demonstrated some functional adaptation to plant habitat of those microbes (Levy et al. 2018; Levy et al. 2018). For example, numerous functions involved in plant-microbe interactions were enriched and diversified for plant-associated bacteria in comparison to that of free-living bacteria, including secretion systems such as T3SS and T6SS, carbohydrate metabolism and transport genes, aromatic compound degradation, siderophore biosynthesis, chemotaxis, and flagellum biosynthesis (Levy et al. 2018; Levy et al. 2018).

The rhizosphere microbiome was the earliest and most investigated compartment due to its ecological and agricultural importance, by function as the crucial interface mediating plant water absorption and nutrient uptake, and curating host-microbe communications (Hartmann et al. 2008; Hiltner 1904; Berg et al. 2014). Endosphere microbes are a more taxonomically restricted subset of microbes capable of inhabiting roots, developing a more stable and mutualistic relationship with the plant host (Edwards et al. 2015; Lundberg et al. 2012; Lebeis et al. 2015). Understanding how rhizosphere and endosphere root microbiomes respond to plant development is crucial to leverage their versatile benefits for plant host, and to advance our next green movement in sustainable agriculture (Jez et al. 2016; Bakker et al. 2018; Pieterse et al. 2016). Although the mechanisms underlying root microbiome assembly are not fully understood, advances in next-generation sequencing technology and associated computational techniques have greatly accelerated our understanding of the main orchestrating forces, including soil background (both physio-chemical environmental condition and indigenous microbe pool), plant genotypes/development/activities, and microbe-microbe interactions (Müller et al. 2016; Philippot et al. 2013; Bulgarelli et al. 2013). Root exudates profiles, secondary metabolites biosynthesis (e.g, triterpene and scopoletin), and plant defense signaling (e.g., salicylic acid, ethylene, and jasmonic pathways) are important mechanisms mediating the selective recruitment of root microbiome (Huang et al. 2019; Stringlis et al. 2018; Lebeis et al. 2015; Zhalnina et al. 2018).

When a seed germinates, it not only encounters changing soil physio-chemical conditions but is also confronted with diverse microorganisms ranging from detrimental to neutral to beneficial (Beattie 2018). Furthermore, germinating seeds and early-stage seedlings are particularly vulnerable to abiotic and biotic stresses, such as drought and fungal pathogens (Nelson 2018). Consequently, plants have evolved adaptive strategies to ensure their successful transition from seed to seedling. In fact, during the germination and establishment stages, plant roots utilize physiological/morphological plasticities in response to favorable or unfavorable soil conditions (Gruber et al. 2013; Padilla et al. 2007; Robbins and Dinneny 2015). It was recently suggested that the seed-carried microbiome plays a crucial role during plant germination by acting as the initial inoculum for the root microbiome (Martinez-Rodriguez et al. 2019; Nelson et al. 2018; Nelson 2018). In addition, the seed microbiome has profound impacts on plant performance and productivity by functioning as a path for vertical transmission across plant generations (Nelson 2018; Shade et al. 2017; Truyens et al. 2015). However, we still lack information in terms of how robustly these seed-carried microbes are kept within plant roots along with soybean development in comparison to the colonization by the soil-derived microbe.

Fungicide seed treatment has been a very common practice for modern agriculture to eliminate early-stage fungal pathogen infection. The efficacy varies depending on the fungicide type, local climate and pathogen pressure (Solorzano and Malvick 2011; Munkvold 2009). While numerous research studies evaluate the effectiveness of fungicide application in reducing disease severity, increasing plant stands, growth and yield (Solorzano and Malvick 2011; Urrea et al. 2013; Weems et al. 2015), fewer have examined non-target effects on microbes. *In vitro*/microcosm assay-based studies indicated that fungicide seed treatment dramatically inhibited legume nodulation and AMF formation while other suggested insignificant or minor influences depending on their mode of action (Cameron et al. 2017; Muthomi et al. 2007; Kyei-Boahen et al. 2001; Buysens et al. 2015). Considering the unintended but possible adverse effects that fungicide treatments can exert on diverse plant growth-promoting rhizobacteria (PGPR), a taxonomically broad field-based evaluation of associated microbiome is of crucial importance for understanding if there are trade-offs between benefits from chemical-based fungicide and PGPR-based biocontrol applications (Mubeen et al. 2006).

Plant intrinsic development has been widely demonstrated as a pre-programmed factor modulating associated root microbiome assembly, with root exudates being suggested as one of the underlying mechanisms orchestrating the process (Zhalnina et al. 2018; Chaparro et al. 2013). Despite the increasing number of studies characterizing the dynamics of plant microbiome assembly along plant development (Edwards et al. 2018; Chaparro et al. 2014; Zhalnina et al. 2018; Chaparro et al. 2013; Xu et al. 2018), we still lack a comprehensive understanding of this process in the important legume crop, soybean. The most well investigated developmental stages for root microbiomes studies are the transition stages from seedling to vegetative, and then to reproductive maturity. To expand our understanding of plant microbiome dynamics specifically focusing on the early seedling stages, our study investigated the soybean root microbiome dynamics between week1 and week4 post sowing. We utilized field-grown plants, a randomized plot design, and a statistically robust number of biological replicates to infer robust patterns relevant to soybean agriculture.

4.2 Methods and materials

All samples were collected from a stem canker field trial being conducted at the West Tennessee Research and Education Center in Milan, Jackson (University of

Tennessee, Institute of Agriculture). The field plots were arranged as a completely randomized design (Fig.4.1). In total, 15 plots were selected for this experiment. Plots were sowed with soybean seeds treated with different fungicide treatments: Cruiser Maxx (CM), EverGol Energy (EE) or non-fungicide control (CT) with five replicated plots for each fungicide treatment. The ingredients information was illustrated in Table S4.1. The soybean seeds (Armor DK 4744 variety) were collected from a former foliar fungicide treatment trial which got severe stem canker disease, and the seeds were confirmed being infected with stem canker pathogen. Fungicide treatment were conducted ‘in house’ using a Hege 11 Liquid Seed Treater. When two or more fungicides were applied, the fungicides were mixed and used as a single application (Rothrock et al. 2004). Fungicide treatments were mixed with Color Coat Red (1 oz/cwt) in a total slurry rate of 30 oz/cwt (i.e. the amount of water added to each treatment was adjusted to have a 30 oz/cwt slurry).

The day before sowing soybean seeds, fresh soil samples from each of the plots were collected from six sites per plot using a soil probe. The six fresh samples from a single plot were homogenized as a composite sample representing that plot. One week, 3 weeks and 4 weeks after sowing soybean seeds, soybean roots and tightly attached soil were collected from six soybean seedlings within each plot and pooled together as a composite sample at each time (Fig. 4.1). Briefly, soybean seedlings from the two edge rows were gently removed from the soil, with loosely attached soil being discarded and tightly attached soil together with soybean roots removed from the aboveground part of the plant. The attached soil and roots put into a 50mL centrifuge tube filled with 30mL phosphate buffer (per liter: 6.33 g of $NaH_2PO_4.H2O$, 16.5 g of $Na_2HPO_4.7H2O$, 200 µL Silwet L-77). At the same time, a bulk soil sample was taken using a soil probe from the between row area in the adjacent to the soybean roots collection areas. The soil samples from the same plot were mixed as a composite bulk soil sample. All soil and root samples were immediately put onto the ice and stored in a cooler before brought to the lab for further processing. Rhizosphere soil pellet collection and separation of soybean root samples were processed following the Lundberg et al. (2012) protocol (Lundberg et al. 2012). To ensure consistent sample processing, soil from the field prior to sowing and bulk soil samples during the trial were processed in parallel. A similar amount of bulk soil was collected to approximately match the volume of rhizosphere soil samples. All samples were placed into the same volume of phosphate buffer. Collected soil pellets and root tissue were stored at -80℃ before further processing.

DNA extraction from soil samples used the DNeasy PowerSoil kit from QIAGEN. Root samples were freeze-dried using a Labconco freeze dry system. Those dry root samples were grounded to a fine powder using a Mixer Mill, and DNA extracted using the same kit. Extracted DNA was quantified using a Nanodrop with a concentration around 30ng/uL for soil samples and 100ng/uL for root samples. To characterize the seed microbiome, DNA was extracted from soybean seeds with CM, EE, and CT fungicide treatments. No surface sterilization of soybean seeds was performed in order to ensure treatment consistency between those planted in the field and those used for seed microbiome examination. Before DNA extraction, seeds were soaked using phosphate buffer overnight, and mortars and pestles were autoclaved. Then soybean seeds were grounded to a fine powder, and the slurry was allocated to DNA extraction buffer and extracted following the same protocol.

16S rRNA ribosome amplicon sequencing was used profile bacterial community using the Illumina MiSeq platform and targeting the V3-V4 region with specific primer sets (forward primer 341F= 5′-CCTACGGGNGGCWGCAG-3′ and reverse primer 785R = 5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al. 2013). The library was prepared with two PCR steps. During the first PCR, amplicon specific primer sets were appended with adapter overhang sequences compatible with Illumina Nextera XT index sequence using 2X KAPA HiFi HotStart ReadyMix. The PCR cycle was set up as: 95°C for 3 minutes; 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes. The PCR product was then purified with AMPure XP beads. During the second step of PCR, Illumina dual-index barcodes together with Illumina sequencing adapters were ligated to the amplicon products from step one using the Nextera XT Index Kit with PCR cycle: 95°C for 3 minutes; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes. To eliminate the amplification of plant chloroplast and mitochondria, peptide nucleic acid (PNA), including the anti-mitochondrial PNA (mPNA) 5′-GGCAAGTGTTCTTCGGA-3′ and the anti-plastid PNA (pPNA) 5′-GGCTCAACCCTGGACAG-3′ were used to block elongation during the first step of PCR (Lundberg et al. 2013). The prepared 16S rRNA amplicon library was sequenced using MiSeq v3 to yield 2X300bp paired reads.

Similarly, the fungal community was characterized by amplicon sequencing using ITS2 (internal transcribed spacer 2) specific primer sets. ITS based fungi profiling was accomplished with MiSeq 250bp paired-end sequencing using primers with a mixture of

6 forward and 2 reverse primers at an equal concentration (Cregger et al. 2018), which are designed to allow better detection of *Chytridiomycota*, *Sebacinales*, *Glomeromycota*, *Sordariales*, *Stramenopila* and *Archaearhizomycetes*. Library preparation followed the same protocol as above. For seed samples and root samples, a large percentage of reads were classified as soybean ITS2 sequences after a preliminary test of sequencing. Consequently, an additional PNA blocker (ATCTGGGTTCATGGCCG) was designed and used to eliminate the amplification of soybean ITS2 sequences, which could be found in Github (https://github.com/liufangbaishikele/Soybean_rhizosphere_microbiome/blob/master/2018_fungicide/Seed_treatment/ITS/PNA_design_doc.md).

The quality of raw reads was evaluated with fastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Mothur software was used to process the sequencing data. Commands and parameters are documented in GitHub (https://github.com/liufangbaishikele/Soybean_rhizosphere_microbiome/tree/master/2018_fungicide/Seed_treatment) (Kozich et al. 2013). The ITS2 read quality was slightly degraded in the primer region only. To resolve this, ambiguous nucleotides along the primer regions were allowed for sequencing assembly in mothur using *make.contigs*; this is not expected to impact results as the primer sequence is removed prior to clustering and taxonomic assignment. Briefly, paired reads were assembled and resulting contigs with low quality were filtered out. Unique sequences were aligned to each other using SILVA 132 as the reference (Quast et al. 2013). Preclustering was used as a denoising strategy by accommodating a sequencing error of 1 base pair of difference along with every 100 base pairs of length. The vsearch tool was used to detect and remove chimeras based on the UCHIME algorism (Rognes et al. 2016). A naive Bayesian classifier was used to classify each sequence against the SILVA taxonomy reference. After filtering out mitochondria, chloroplast, and Archaea sequences, remaining sequences were clustered into OTUs (operational taxonomic unit) at a threshold of 97% of similarity, with corresponding OTUs classified down to genus level. For ITS2 sequencing data, assembled contigs were clustered using de novo OTU picking at 97% sequence similarity, and OTUs were taxonomically classified using UNITE as reference (Nilsson et al. 2019; Almario et al. 2017). Similar to the 16S rRNA pipeline, all non-fungi sequences were removed before cluttering into OTU.

To eliminate the potential bias caused by the varied sequencing depth between samples, all samples were rarefied to the minimum sequencing depth of 13,021 for 16S rRNA sequencing data across all samples (except those of seed) and singletons were removed before used to downward community analysis. Due to lower sequencing depth than other samples, seed samples were analyzed independently without rarefaction. ITS sequences were not rarified due to relatively low depth. Community analysis was conducted in R using Phyloseq, ggplot2, reshape2, dplyr, vegan, permute and stringr packages (R Development Core Team and Others 2011). The Shannon diversity index was calculated and compared between treatments using either ANOVA or Kruskal Wallis variance analysis, followed by Post-hoc or Dunn test for pairwise comparison (as specified in results for each test). A Bray-Curtis distance matrix was calculated to infer community dissimilarity between samples then visualized using a principal coordinate analysis (PCoA) plot. PERMANOVA (permutational multivariate analysis of variance) was used to test treatment impacts based on a permutational test using between sample distance matrix, with permutation being constrained using the permute package.

To infer differentially enriched or depleted bacteria and fungi between compartments, *Lefse* was used to conduct differential abundance analysis under one against all modes (i.e., one taxon is considered to be significantly different only when it is significantly different against all remaining treatments) (Segata et al. 2011). A Mothur command- *make.lefse*- was used to generate the lefse input file using a phylotype-clustered OTU table, associated with the taxonomy table and the metadata. Any taxa with an α less than 0.05 and an LDA score larger than 4 were defined to be significantly different between treatments based on 200 bootstrap iterations. When comparing seed microbiomes and endosphere microbiomes, the relative abundance count table before rarefaction was used as input to generate the lefse input file due to the insufficient sequencing depth for seed data. Differential abundance between compartments was conducted for each individual development stage and differential abundance between development stages was conducted for each compartment.

16S ribosomal RNA sequencing data were mapped to bacterial function and metabolic pathway databases to predict bacterial function using PICRUST2 (Douglas et al. 2019). The most updated KEGG pathway database was used for PICRUST2. The function prediction analysis was done following PICRIST GitHub documentation (https://github.com/picrust/picrust2). Specifically, the mothur function *get.oturep* was used to generate a fasta-formatted sequence file including representative sequences for each OTU, which were subsequently edited to match with the rarefied OTU table. Then the PICRUST full pipeline command was used to conduct function prediction. Next, metaCyc pathway abundance output was used for differential abundance analysis with the DESeq2 package. Pairwise comparisons between each pair of the 11 subsets (ie., Soil, Bulk_wk1, Bulk_wk3, Bulk_wk4, Rhi_wk1, Rhi_wk3, Rhi_wk4, Endo_wk1, Endo_wk3, Endo_wk4 and SEED) were conducted to infer pathways that were significantly enriched or depleted between treatments. Pathways consistently and significantly enriched/depleted between compartments were subtracted from the original pathway dataset and used for visualization by the heatmap plot.

4.3 Results

4.3.1 Sequencing data summary

After sequencing, we captured 20,837,263 reads for 16S rRNA gene sequencing and 25,378,396 reads for ITS2 amplicon sequencing across 64 samples. For 16S rRNA sequencing reads, the median quality score for the first 270bp and 250bp was larger than 34 for forward and reverse reads respectively. In terms of ITS2 sequencing reads, most of the bases yield a quality score larger than 34 for both forward and reverse reads except for the primer region. After filtering low quality reads, chimera and non-bacteria and non-fungi sequences13,079,622 and 4,849,883 informative reads remained for 16S rRNA and ITS2 sequencing data.

4.3.2 Fungicide seed treatment impact on root bacterial community was undetectable

Considering the high heterogeneity of soil indigenous microbial communities within a field, it is necessary to ensure the randomization of the plot design. For this purpose, we collected composite fresh soil samples from each plot before sowing seeds, and compared the bacteria composition differences between plots assigned with a different fungicide treatment. The PERMANOVA analysis results suggested a well-randomized assignment of fungicide treatments across different plots (PERMANOVA, $F_{(2,12)} = 1.03$, $p = 0.38$). After confirming the randomization of plot design, we tested the impact of fungicide seed treatment on the taxonomic makeup of the root-associated bacterial community using soybean development, compartment and read-depth factors. Fungicide application was insignificant in the overall analysis (PERMANOVA, $F_{(2,126)} = 1.79$, $p = 0.06$) and insignificant within each compartment: bulk (PERMANOVA, $F_{(2,39)} = 1.30$, $p = 0.06$), rhizosphere (PERMANOVA, $F_{(2,38)} = 1.32$, $p = 0.20$), and endosphere (PERMANOVA, $F_{(2,39)} = 1.38$, $p = 0.20$). We also tested the fungicide impact within each combination of compartment and development stage as well as seed bacteria. The only significant impact was found for the seed bacterial microbiome (Table 4.1). For seed bacteria, both CM and EE fungicide seed treatments significantly decreased the relative abundance of *Actinobacteria*, while *Firmicutes* relative abundance was increased.

4.3.3 Distinct bacterial microbiome composition between compartments

Bacterial composition was revealed to be significantly different between the compartments assessed in this experiment: soil prior to sowing, seed, bulk soil, soybean rhizosphere, and soybean endosphere (PERMANOVA, $F_{(4,159)} = 42.85$, $p < 0.001$***). Between compartment differences explained 51.87% of all between sample variation. The bacterial composition between pre-sowing soil resembled bulk soil, both of which were evidently divergent from those of rhizosphere, endosphere and seed microbiomes (Fig. 4.2 and Fig. S4.1). To investigate how compartment-specific communities changed along soybean development stages, we subset samples based on development stages and conducted PERMANOVA analyses. The results revealed comparable compartment effects between week1, week3, and week4, explaining 53% to 64% of between samples variation. Week4 exhibited the highest compartment impacts in comparison to week1 and week3 (Table 4.2 and Fig. S4.2A-C). The read_depth impact was significant for week 3 samples when tested as the only factor (PERMANOVA, $F_{(1,43)} = 6.10$, $R^2 = 12.42\%$, $p = 0.001$), but this read_depth effect was no longer significant when its marginal effects were tested together with the compartment factor. Detailed between sample dissimilarity comparison results suggested that bacterial community composition was more divergent between bulk soil and rhizosphere for one-week old soybean seedlings (Fig. 4.3). However, this dissimilarity greatly decreased at later time points. In contrast, the bacterial community composition was more similar between the rhizosphere and endosphere at week1 compared with that of week3 and week4. Endosphere bacterial composition were more similar across different plots at week3 and week4 in comparison to week1.

At the phylum level, we observed significant between-compartments changes in bacterial relative abundance (Fig. 4.4A). More specifically, bacterial phyla including *Acidobacteria*, *Verrucomicrobia*, *Gemmatinomadetes*, and *Planctomycetes* phyla were gradually reduced from surrounding soil to rhizosphere and then to endosphere. In contrast, *Proteobacteria*, *Firmicutes*, and *Actinobacteria* were highly enriched from surrounding soil toward soybean roots. Inside of soybean seed, the bacterial community was dominated by *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Cyanobacteria*. No significant differences were found in alpha diversity between pre-sowing and bulk soil samples, both of which harbor significantly more diverse bacterial communities in comparison to the rhizosphere, endosphere and seed samples (Kruskal-Wallis chi-squared = 132.36, $p < 2.2e-16$) (Fig. 4.5A). The lowest diversity compartments were the root endosphere and soybean seed, which shared a comparable bacterial Shannon diversity score.

4.3.4 Plant development impacts on bacterial community composition and diversity

From the aforementioned results, we found that soybean nurtured a very specialized microbial community across compartments as a result of selective enrichment/depletion of a subset of microbial consortia. As plant development has been widely recognized as a crucial factor modulating root bacteria assembly, we further characterized soybean compartment-specific bacteria dynamics along with soybean development. Unsurprisingly, bacterial community compositions were significantly different between week1, week3 and week4 time points (Fig. S4.2D-F) for all three compartments (i.e., bulk soil, soybean rhizosphere, and endosphere). More intriguingly, this between development differences of the bacterial community increased significantly from bulk soil to rhizosphere and then to endosphere as indicated by the increasing variation that could be explained by the time factor (Table 4.3). In contrast, variation in the bacterial community between plots was significantly reduced from bulk soil to soybean rhizosphere and endosphere (Table 4.3).

Likewise, the bacterial Bray-Curtis dissimilarity dot plot suggested increasing development impacts from bulk soil to rhizosphere and endosphere (Fig. 4.6). Between plots dissimilarities varied within a similar range for pre-sowing soil and bulk soil community. Bacterial community dissimilarity was comparable between bulk soil samples across time. For both the rhizosphere and endosphere bacterial community, the Bray-Curtis dissimilarities were larger for those between week1 and week3, and those between week1 and week4 in comparison to those between week3 and week4. Both of the above findings indicated that an intrinsic selection of bacteria consortia that occurred in the soybean rhizosphere and endosphere along soybean development despite the different indigenous microbiome composition in the surrounding soil.

In addition to the compositional differences, we also observed distinct changes in bacteria alpha diversity. The week1 rhizosphere bacterial community was less diverse in comparison to week3 and week4 (Fig. 4.5B). In contrast, endosphere bacteria were more diverse at week1 compared with week3 and week4, and seed bacteria diversity was very comparable with that of endosphere at week3 and week4. However, we did not observe significant bacterial diversity difference between collection events when bulk soil was compared.

4.3.5 Dynamic recruitment of bacteria consortia into each compartment during soybean development

To dissect the dynamic recruitment of individual bacterial taxa between compartments and along with developments, we conducted differential abundance analysis across all taxonomic levels. Between compartments, the recruitment/depletion pattern was compared separately at each development stage (Fig. S4.3). The results suggested that some bacterial taxa were consistently enriched into the specific compartment despite soybean development (Fig. 4.7). Specifically, *Acidobacteria*, *Gemmatimonadetes*, *Verrucomicrobia*, *Planctomycetes*, and *Deltaproteobacteria* were consistently depleted in both rhizosphere and endosphere compartments. In contrast, *Gammaproteobacteria*, *Betaproteobacteriales*, and *Burkholderiaceae* were robustly enriched in the soybean rhizosphere, while *Alphaproteobacteria*, *Rhizobiales*, *Actinobacteria*, *Streptomycetales*, *Streptomycetaceae* and *streptomyces* were consistently enriched in soybean endosphere. In addition to the aforementioned consistent selection on specific bacteria taxa, some other bacteria were selectively enriched into the rhizosphere or endosphere at specific developmental stages. For example, *Firmicutes* (including *Bacilliaceae* and *Paenibacillaceae*) and *Solirubrobacteriales* were selectively enriched in the soybean endosphere at week one, while *Proteobacteria*, *Xanthobacteraceae*, *Bradyrhizobium*, *Novosphingobium*, and *Sphaerisporangium* were discriminately enriched in endosphere at week3 and week4. This development-specific enrichment of bacteria was more divergent in the soybean rhizosphere. *Ralstonia*, *Acinetobacter*, and *Moracellaceae* were specifically enriched in the soybean rhizosphere at week one, while *Sphingomonadaceae*, *Pseudomonadaceae*, and *Nitrosomonadaceae* were selectively enriched into rhizosphere either at week3 or week4.

To provide a comprehensive picture of the dynamic changes for each bacterial taxa, we conducted differential abundance analysis between development stages for each compartment separately (Fig. 4.8 and Fig. S4.4). For bulk soil samples, development impacts on individual taxa were very limited, within few taxa including *Gammaproteobacteria*, *Sphingomonas*, and *Bacteroidetes* being exclusively enriched at week3. In contrast, the dynamics of individual bacteria taxa were very prominent within the rhizosphere and endosphere compartment. In Endosphere, the relative abundance of *Bradyrhizobium*, *Sphaerisporangium*, *Novosphingobium* genus were significantly enriched from week1 to week3 and week4, while, *Streptomyces*, *Ralstonia*, *Bacillus*, and *Paenibacillus* were significantly decreased. In the rhizosphere, *Novosphingobium* and *Sphingomonas* were significantly accumulated at week3 and week4 compared with week1, while *Burkholderiaceae* (especially *Ralstonia*), *Enterobacteriaceae* and *Pseudomonadiale*s (specifically *Acinetobacter*) were significantly reduced after one week. In addition, *Acidobacteria*, *Verrucomicrobia*, *Planctomycetes*, and *Gemmatimonadetes* phyla were significantly reduced in the rhizosphere in comparison to bulk soil community at week1, whose abundance then increased at week3 and week4 resulting more similar composition as bulk soil community.

4.3.6 Early soybean seedling endosphere bacteria were mainly recruited from the rhizosphere

To infer the potential source for endosphere bacteria assembly, we generated a Venn plot between rhizosphere, endosphere and seed microbiome using bacteria genera information (Fig. 4.9). The endosphere microbiome was mainly a subset of bacteria of the rhizosphere (i.e., week1=85.78%, week3=93.67%, and week4=93.21%), within which about 78% of the bacteria exclusively overlapped with the rhizosphere compartment, while the other 22% overlapped with both the rhizosphere and seed microbiome. In contrast, only about 2% of the endosphere microbiome exclusively overlapped with the seed community. The seed microbiome was largely represented as a subset of the endosphere microbiome, with only about 5% - 10% of taxa being unique to the seed. At the genus level, the seed bacterial community was dominated by *Pantoea*, *Oxyphotobacteria_unclassified*, *Methylobacterium*, *Pseudomonas*, *Acinetobacter*, which together accounted for 37% of the total bacterial community. However, these seed dominant bacteria dramatically decreased in one-week-old seedling roots, which were dominated by *streptomycetes*, *Streptomycetaceae_unclassified*, *Enterobacteriaceae_unclassified*, *Bacillus*, *Enterobacter* at week1 (Table S4.2).

4.3.7 Distinct bacterial metabolic pathways between compartments and along with soybean development

The bacterial function was predicted based on 16S taxonomy and abundance information, which revealed a very distinct pathway profile between bulk, rhizosphere, and endosphere as well as seed compartments (Fig. 4.10). Overall, the seed microbiome featured with limited function potential compared with bulk soil and root-associated bacterial community. Bacterial functional potentials were revealed to be comparable between pre-sowing soil and bulk soil, which shared a majority of pathways with rhizosphere week3 and week4 samples, with pathways involved in fermentation, nucleoside and nucleotide biosynthesis, sugar nucleotide biosynthesis, autotrophic CO2 fixation and denitrification greatly enriched in comparison to endosphere microbiome and rhizosphere week1 microbiome. In contrast, pathways belonged to aromatic compound degradation, secondary metabolites degradation and detoxification were more highly selected for the endosphere microbiome as well as rhizosphere week1 microbiome. Specifically, bacterial secondary metabolites degradation pathway (e.g., D_galacturonate, myo-inositol degradation) and toluene degradation were more abundant in rhizosphere week1 in comparations to week3 and week4. For endosphere bacteria, the week3 and week4 communities featured more diverse aromatic degradation pathways in comparison to week1.

4.3.8 Significant fungicide impact on soybean rhizosphere fungal community at week3

Randomization of plot design was tested using the pre-sowing fungal community, which suggested the selection of a well-randomized plot for fungicide seed treatment (PERMANOVA, $F_{(2,12)} = 1.04$, $p = 0.359$). The impact of fungicide application on soybean root-associated fungal community was tested using PERMANOVA analysis. The fungicide seed treatment only significantly impacted the rhizosphere fungal community at week3 (Table 4.1). Meanwhile, a marginal ($0.05 < p < 0.1$) fungicide impact was detected for rhizosphere week4, endosphere week3, and the seed community. Differential abundance analysis for fungicide seed treatment was conducted for the rhizosphere week3 fungal community. The results indicated that the genus Chaetomium was significantly decreased in fungicide treated soybean rhizosphere compared with the control soybean (Fig. 4.11). In contrast, *Clonostachys rosea* species was more abundant in Energy Evergol treated soybean rhizosphere.

4.3.9 Significant but minor compartment impacts on fungal community compared with the bacterial community

Fungal community composition differed significantly between soil, seed, rhizosphere and endosphere compartments (PERMANOVA, $F_{(4,159)} = 28.55$, $p < 0.001$***), with 41.80% variations being explained by compartment factor (Fig. 4.12). Similar to the bacteria community, fungi composition was similar between pre-sowing soil and bulk soil. In addition, the rhizosphere fungal community was less different from the bulk soil community in comparison to the bacterial community. Between compartment differences of the fungal community were less evident compared with the bacterial community due to the large between-plots variation (Fig. 4.3). We also compared the compartment impacts within each development stage, which suggested a slightly larger compartment impact at week3 (Table 4.4 and Fig. S4.6A-C). However, the compartment's impact on the fungal community was relatively small compared with that of bacteria (Table 4.1). In contrast, fungal community differences were more explained by the plot, with about 29-33% of community difference able to be attributed to the between plots variation.

The fungi Shannon diversity was significantly between compartments (Kruskal-Wallis chi-squared = 136.17, df = 10, p < 2.2e-16), which gradually but significantly decreased from the pre-sowing soil and bulk samples to rhizosphere and endosphere (Fig. 4.13). Again, we did not observe a significant difference between endosphere and seed fungi diversity. The stacked barplot of fungi classes suggested a gradient increase of *Sordariomycetes* and *Glomeromycetes* classes across the transition from bulk to root endosphere. Meanwhile, *Mortierellomycetes* dramatically reduced in the soybean endosphere. Within soybean seed, the fungal community was dominantly colonized by *Sordariomycetes* and *Dothideomycetes* (Fig. 4.14A).

4.3.10 The impact of soybean development on fungi composition was similar between different compartments

For the fungal community composition, a relatively large amount of variation was explained by between-plot differences, while the impact of soybean development (i.e. time) was relatively small. In addition, we observed a slightly decreasing development impact on the fungal community from the bulk soil to the rhizosphere, and then to the endosphere compartment (Table 4.5 and Fig. S4.6D-F). In bulk soil, fungal community composition was very different at week3 compared with week1 and 4. Within the rhizosphere, the fungal community was more similar at week3 and week4 in comparison to that of week1. Inside of the soybean endosphere, development impacts were less evident, but the week1 community was still divergent from week3 and 4. The decreasing development impact on the fungal community was also reflected in the Bray-Curtis dissimilarity dot plot (Fig. 4.6). On average, the between plot (i.e., within weeks dissimilarity) dissimilarity of the fungal community increased from bulk to endosphere compartments. Contrarily, this between plots dissimilarity was dramatically decreased for bacteria community when moving from bulk soil to endosphere despite a similar between plots variation for pre-sowing bacterial community and pre-sowing soil fungal community. For fungal community diversity, we did not find any development impact (Fig. 4.13).

4.3.11 Differentially enriched/depleted fungi taxa between compartments and soybean development stages

Despite the high between plots variation for the fungal community, we observed compartment-specific recruitment on individual fungi taxa (Fig. 4.15, and Fig. S4.7). The *Chaetomium* genus was uniquely enriched in the soybean rhizosphere, while the *Fusarium* genus (especially *Fusarium solani* species) was selectively enriched in the soybean endosphere. In addition to this consistent selection between compartments, few fungi taxa were selectively enriched into a specific compartment depending on the specific development stage. For example, the *Gigaspora* genus (specifically *Gigaspora margarita*) was significantly enriched in the soybean rhizosphere at week4 but not week1/3, while, the *Pyxidiophora* genus was selectively enriched in the soybean rhizosphere at week1 and week3 instead of week4. Similarly, the *Branch06* and *Gigasporaceae* families were selectively enriched in the endosphere at week1 and week3, while the *Botryosphaeriaceae* family was significantly enriched at week4. In addition to this development specific recruitment of specific fungal taxa, we also observed some within-compartment dynamics for several fungal taxa (Fig. S4.8). Within the soybean endosphere, the relative abundance of the *Gigaspora* genus (specifically *Gigaspora margarita*), and *Fusarium* genus (specifically *Fusarium solani*) significantly decreased in soybean roots along with the development of the soybean. Meanwhile, the relative abundance of the *Botryosphaeriaceae* family was gradually increased from week1 to week4 within the soybean endosphere. In the soybean rhizosphere, the *Gigaspora* genus was significantly enriched at week4 compared with week1/3. In contrast, the *Bionectriaceae* and *Chaetomiaceae* families were significantly more abundant in week3/4 in comparison to that of week1. In bulk soil, both the Fusarium and Chaetomium genus were more abundant at week4 compared with week1/3. The abundance of fungal Dothideomycetes class was significantly larger in week3 compared with week1/4, which is not the case for either the rhizosphere or the endosphere fungal community.

4.3.12 Seed fungal community composition and its relative contribution to endosphere fungi assembly versus to that of rhizosphere

Seed fungi taxa were mainly overlapped with that of the rhizosphere and endosphere, which accounted for 86%-88% of the seed fungi composition (Fig. 4.16). In addition, more fungi taxa were found to be seed-specific (ranging from 11.73% to 13.68%) in comparison to that of the bacterial community (ranging from 5.55% to 10.72%). Similar to the composition of the bacterial endosphere community, the soybean endosphere fungal community was also highly overlapped with that of the rhizosphere (i.e., week1=88.98%, week3=89.30%, week4=94.96%). Meanwhile, we noticed that there were about 48.22%-52.96% of the endosphere fungal taxa were exclusively overlapped with rhizosphere, while only 0.84%-1.47% was exclusively overlapped with seed fungal composition. The seed fungal community was dominated by unclassified Nectriaceae taxa, which accounted for 37% of the total abundance (Table S4.2). At week1, the endosphere fungal community was also dominantly composed by Nectriaceae (40%), however, most of them were classified as Fusarium solani. Another two dominant fungi taxa within the seed community, i.e., *Cercospora* (10.25%) and unclassified Diaporthales (18.03%), were dramatically low in soybean endosphere at week1.

4.4 Discussion

4.4.1 Fungicide impacts on soybean root bacterial and fungal microbiome

Fungicides seed treatment has been widely applied to protect seed from pathogen infection prior to or during seed germination, with varied improvements on plant growth or yield depending on the mode of action of the fungicide and local climate conditions (Jin et al. 2013; Munkvold 2009). This inconsistency may be partially explained by non-target effects on beneficial partners of the plant, such as rhizobia and arbuscular mycorrhizal fungi as well as PGPRs (Muthomi et al. 2007; Kyei-Boahen et al. 2001; Cameron et al. 2017). In this study, we characterized global taxonomic changes of soybean bulk, rhizosphere and endosphere microbiome compositions in response to the applications of two seed coated fungicide formula, i.e., Cruser Maxx and EverGol

Energy. Looking at early development time points, the results suggested that the fungicide seed treatment effect was insignificant for soybean bacterial root microbiome assembly. In contrast, significant though minor changes of the fungal community were observed for the rhizosphere community at week3, with *Chaetomiaceae* (specifically *Chaetomium*) being significantly decreased under both CM and EE fungicide treatments. Several *Chaetomium* strains are characterized as biocontrols for pathogens and thus may be providing benefits to plant health (Tveit and Wood 1955; Soytong et al. 2001). We also found that a potential biocontrol fungi species, *Clonostachys rosea*, were significantly enriched under EE fungicide treatment. Consistent with Cameron et al. study, we found no impact of fungicide seed treatment on AMF abundance (Cameron et al. 2017). This may be related to the application dosage compared with former studies. Alternatively, seed fungicide treatment impacts may be more evident when applied under higher pathogen pressures (Solorzano and Malvick 2011). Overall, our evaluation of fungicide treatment did not indicate a strong non-target effect on rhizobia nor AMF. However, conclusions based on one season of results is not sufficient to draw conclusions in terms of long-terms ecological or agricultural impacts.

4.4.2 Compartment specific recruitment of root-associated microbiome

In general, soybeans develop compartment-specific microbiomes as reflected by the distinct bacterial and fungal community composition moving from high diversity in the surrounding soil, to less diversity in the rhizosphere, and least in the endosphere. The relative abundance of *Acidobacteria*, *Verrucomicrobia*, *Gemmatimonadetes*, *Planctomycetes*, and *Deltaproteobacteria* dramatically decreased in the soybean rhizosphere while *Gammaproteobacteria* abundance was significantly increased, which is consistent with former reports about the soybean root microbiome (Liu et al. 2019; Xiao et al. 2017). In comparison to the rhizosphere bacterial microbiome, *Actinobacteria* and *Firmicutes* were enriched within soybean roots and seed microbiome. This dramatic enrichment of *Actinobacteria* has been noted in roots from other plant species and may indicate some metabolic adaption of this bacteria genus within root microenvironment or attributed by its strong competition capacities as a result of its diverse production of antimicrobial metabolites (Bulgarelli et al. 2012; Edwards et al. 2015; Lundberg et al. 2012).

The soybean seed bacterial microbiome is a subset of endosphere bacteria, mainly consisting of *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Cyanobacteria*. At the genus level, *Pantoea*, *Pseudomonas*, *Methylobacterium* and *Acinetobacter* are the most abundant consortia inhabiting either within or on the surface of soybean seed, with the first two genera being consistently reported as predominant taxa within seeds across several plant species (Chen et al. 2018; Truyens et al. 2015; Rezki et al. 2018; Barret et al. 2015). Seed-isolated bacteria including *Pantoea* strains featured with high tolerance to high osmotic pressure, which may indicate an adaptive evolution in response to the increased osmotic pressure during seed maturation (Mano et al. 2006). In addition, numerous *Pantoea* strains have probiotic traits, including nitrogen fixation, mineral phosphate solubilization, auxin production, and chitinase secretion as well as anti-fungi activities (Ruiza et al. 2011). Similarly, *Pseudomonas* strains are recognized for their plant growth-promoting features and widely applied as biocontrol agents (Weller 2007). Despite the low relatively small contribution of the seed microbiome to the establishment of the early seedling microbiome, some of the specific seed-carried microbes have features that may be beneficial to the germinating plant (Bewley 1997).

4.4.3 Dynamic root microbiome structure along with soybean development

Despite the compartment-specific recruitment of bacteria taxa from the surrounding soil, we detected evidently dynamic changes of specific microbial taxa in the soybean rhizosphere and endosphere along with the soybean development. Although between collecting times difference were detected for bulk soil bacterial microbiome, this time effect was little in comparison to that of rhizosphere and endosphere. At week1, soybean displayed with evident rhizosphere effects with significantly decreased the abundance of *Acidobacteria* and dramatic accumulation of *Gammaproteobacteria* (specifically *Enterobacteriaceae* and *Burkholderiacea*) together with a significant reduction of bacterial diversity in comparison to surrounding bulk microbiome. Consistently, *Burkhoderiales* are earlier colonizer found in rice research too (Edwards et al. 2018). However, this rhizosphere effects diminished when the plant grows older to week3/week4 and the relative abundance *Acidobacteria*, *Verrucomicrobia*, *Gemmatimonadates* re-enriched, and *Gammaproteobacteria* reduced to a similar level alike those of bulk soil. Consequently, when evaluating the degree of rhizosphere effects among plant species, we may want to make sure the investigated plants were under similar development stages as chronological age is less consistent compared with developmental ages (Edwards et al. 2018).

Numerous studies reported dynamic changes of rhizosphere bacterial community composition along with plant development and associated it with the dynamic changes of root exudates profiles (Chaparro et al. 2013; Zhalnina et al. 2018; Xu et al. 2018; Shi et al. 2015). For example, sugar and sugar alcohol highly accumulated during the early development of Arabidopsis, while the secretion of phenolics and amino acids were increased over development, both of which were demonstrated to correlate with the functional capacity of responding microbe (Chaparro et al. 2013). Similarly, the dynamics of sorghum rhizosphere microbes were attributed to their divergent metabolic preference toward the preprogrammed root exudates profile (Zhalnina et al. 2018). Due to the different stages, each study focused on and the host plant investigated, we did not find reports about these distinct early seedling development changes of the rhizosphere bacterial community as revealed in our study. However, *Acidobacteria* relative abundance in Arabidopsis rhizosphere bacterial community exhibited similar dynamics as observed in our study, which presented an increased abundance of *Acidobacteria* when plant developed from seedling to vegetative stages, which were then increased when the plant grew to the bolting and flowering (Chaparro et al. 2014).

To thrive under the continuous attacking from a pathogenic microorganism, physically vulnerable root tissues have evolved with the intrinsic secretion of diverse ‘underground warfare chemicals’ such as phytoalexins, defense proteins or other unknow secondary metabolites (Bais et al. 2006). The dramatic enrichment of *Enterobacteriaceae* and *Burkholderiacea* in the rhizosphere at the early seedling stages may reflect general manipulation strategy during the establishment of seedling which is a critical transition stage determining whether the seed will finish the transition to the next generation or not (Nelson 2018). Specifically, before the roots system developed to be big enough for water, nutrient absorption and pathogen defense, the host plant may need more facilitations supplied by associated organisms. Actually, *Enterobacter* *cloacae* were able to eliminate infection by fungal pathogen *Pythium ultimum sporangium* via various mechanisms (Windstam and Nelson 2008; van Dijk and Nelson 2000; Kageyama and Nelson 2003). In addition, *Enterobacter* strains could enhance plant osmotic stress tolerances during seed germination and seedling growth (Li et al. 2017). Alternatively, the aforementioned specific rhizosphere bacterial community at week1 may mirror soybean specific developmental needs. As soybean evolved with the symbiotic interaction with nitrogen-fixing rhizobia, it may need to recruit some assistant bacteria for more efficient nodule formation during its early development stages. *Ralstonia solanacearum* were widely distributed devastating soil-borne pathogen featured with a wide host range (Salanoubat et al. 2002). Their high abundance in the soybean rhizosphere at week1 may reflect the vulnerability during the early seedling development stage, which warranted for further detailed examination.

Similarly, soybean endosphere bacterial microbiome featured with distinct community composition at week1 compared with week3/4, which mainly attributed to the dramatic enrichment of *Bradyrhizobium* within soybean roots and distinguished decrease of *Streptomycetes* and *Firmicutes* (specifically *Bacillus*) in week3/4. Based on field observation, soybeans usually start to form nodules about 2 weeks after germination. Consequently, it is not surprising to observe the striking enrichment of *Bradyrhizobium* at week3 and week4 as a result of nodule formation. However, it is interesting when you start to think about the question “why nodulation did not initiate immediately after seed germination?”. As mentioned before, the successful establishment of the seedling is crucial for a plant to succeed to the next generation. In addition, early established seedlings are very vulnerable to abiotic and biotic stresses, which make it necessary to set a successful stand as the priority for development. Once seedlings are well established, soybean started to release symbiotic signals to the rhizosphere to specifically attract *Bradyrhizobium* to form nodules. Interestingly, those highly enriched bacteria taxa including Streptomycetes and Firmicutes were revealed as the most strongly enriched bacteria when sorghum were grown under drought (Xu et al. 2018). Additionally, numerous Streptomycetes strains was reported with various PGPR traits ranging from osmotic stress alleviation (e.g., salt and drought stress), fungal pathogen resistance and phosphate solubilization (Jog et al. 2014; de Vasconcellos and Cardoso 2009; Palaniyandi et al. 2014; Mendes et al. 2011). Actually, it has been reported that the co-inoculation of *Streptomycetes* strains and *Bradyrhizobium* japonicum could greatly improve soybean nodulation and growth (Htwe et al. 2019). Similar synergetic enhancement by *Bacillus* strains on soybean nodulation were reported as well (Subramanian et al. 2015). Consequently, the early seedling structuring of related bacterial microbiome warranted for more systemic and mechanistic understanding in order to maximize the expanded benefits.

4.4.4 Relative contributions of rhizosphere or seed microbiota for the establishment of the root microbiome

The undoubted impact of seed microbiome on plant fitness especially during germination and early seedling development has been widely recognized. However, we still lack a comprehensive understanding in terms of the relative importance of seed derived versus soil indigenous microbes during this establishment of early root microbiome. By comparing the composition of the week1 bacterial community with seed microbiome, we found that most of the seed dominant bacteria (*Pantoea*, *Methylobacteria*, *Pseudomonas*, and *Acinetobacter*) dramatically decreased in soybean root microbiome one week after germination. This agreed with former reports showed that when maize were germinated under xenobiotic conditions *Pantoea* tended to be dominant in the rhizosphere, but their abundance decreased dramatically when seeds were growing under the soil with very diverse indigenous bacteria pool (Johnston-Monje et al. 2016). Meanwhile, we did observe some seed microbiome legacy effects on root microbiome at this stage as reflected by the relatively high abundance of *Bacillus* within the root microbiome instead of surrounding bulk or rhizosphere soil, which then distinctly decreased at week3/4. This is consistent with Barret et al. (2015) findings that *Bacillus* remained to be abundant in rhizosphere after 96h of emergency, which was based on in vitro xenobiotic germination assay (Barret et al. 2015). For another *Firmicute* (*Paenibacillus*), we also observed relatively high abundance at week1 instead of week3/4 although we can not rule out the possibility that those *Firmicutes* could be rapidly recruited from surrounding soil as similar pattern were showed for rhizosphere compartment.

Despite increasing studies demonstrating the capacity of seed microbiome to colonize seedling during germination (Barret et al. 2015; Johnston-Monje et al. 2016; Huang et al. 2016), our study demonstrated that after one week of growth, soybean seed derived bacterial microbiome dramatically reduced in root microbiome. This consistency between our findings and former reports could be attributed to the fact that many former reports were concluded based on in vitro/ microcosm study while our findings are based on field study. In this study, we did not characterize the spermosphere (ie., the zone surrounding seed and under the influence of seed carbon deposition), which could reflect the earliest interaction between soil microbe, seed microbes during seed germination (Schiltz et al. 2015; Nelson 2004). The conflicts between out findings and others could be attributed to the different time points being investigated as root microbiome changes along with seed germination (Liu et al. 2012). In fact, Yang et al. (2017) research discovered that dominant bacteria of barley seeds become less abundant within root 8 days after germination, which was more evident when seeds were germinated in the soil in comparison to xenobiotic systems (Yang et al. 2017). Understanding of the relative importance of soil and seed microbiome for the development of root microbiome, and deciphering the dynamics of root and rhizosphere microbiome assembly along seeds germination under more detailed time scale warranted for more attention and efforts considering the critical importance of this development stage and its potential long terms impact on plant performance and fitness.

4.4.5 Compartment specific dynamics of bacterial functional potentials

The meta function of bacterial community were predicted using PICRUST2 packages by linking taxonomy and count information with several function databases including Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs, Enzyme Classification numbers (EC numbers), Clusters of Orthologous Genes (COGs), Protein families (Pfam) and The Institute for Genomic Research’s database of protein FAMilies (TIGRFAM) (Douglas et al. 2019). To make the prediction comparable with shotgun metagenomics output, we chose to present and analyze MetaCyc pathways results, which was further predicted based on EC number prediction with HUMAnN2 algorithms within PICRUST2. To infer the compartment-specific recruitment of associated bacterial function, we conducted differential abundance analysis and generated heatmap based on compartment and development specific pathways. Similar to the compositional separation between samples, we found a distinct pattern of bacterial functional pathways between compartments as well as along with soybean development. Bacterial functional potentials were very similar for bulk soil and pre-sowing soil community. Nutrient and carbon cycling related pathways including fermentation, autotrophic CO2 fixation, denitrification were more abundant for soil inhabit bacterial communities compared with that of endosphere as well as rhizosphere week1 communities. This diverse carbon and nutrient-cycling related pathway reflected the diverse bacteria composition within the soil. Benzoate and crotonate fermentation pathways were formerly discovered involved in syntrophic carbon metabolism, which featured extremely high energy conservation efficiency as well characterized in *Syntrophus aciditrophicus* strain (McInerney et al. 2009; McInerney et al. 2007). In addition, bacteria functions involved in nucleoside, nucleotide, and sugar nucleotide biosynthesis were comparatively richer for the soil bacterial communities. Consistent with bacterial community composition, corresponding metabolic profiles showed similar patterns for the soybean rhizosphere community at week3/4.

Rhizosphere bacteria function for the one-week old soybean seedlings were more like those of the endosphere community. In addition to the dramatic enrichment of pathways related to aromatic compound degradation, several secondary metabolites degradation pathways, including myo- chiro- and scyllo- inostitol and D-galacturonate degradations were highly enriched in week1 rhizosphere in compare to endosphere community. D_galacturonate is the main monomeric constituent of pectin, which is one of the primary components of seed mucilage during germination (Western 2012). This high degradation pathway for the week1 rhizosphere bacterial community may reflect some legacy impact remained even one week after seed germination. Myo-inositol as one type of sugar alcohol were revealed to be highly secreted in Arabidopsis early development time points (between 7-10 days), this high Myo-inositol degradation activity may be related to soybean root exudates as well (Chaparro et al. 2014). In addition, Myo-inositol was indicated to enhance the production of streptomycin by Streptomycetes, which is in accordance with the high abundance of Streptomycetes within root at week1 (Majumdar and Kutzner 1962). Interestingly, this pathway of *myo*-inositol degradation appears to be important in root nodule colonization and competition in rhizobial symbiotes (Galbraith et al. 1998; Jiang et al. 2001). The high abundance of toluene degradation in the soybean rhizosphere at week1 agreed with Chaparro et al. (2014) findings, which revealed significantly enriched expression of bacterial transcripts involved in toluene degradation (Chaparro et al. 2014).

Obviously, metabolic pathways involved in aromatic compound degradation were strikingly enriched for soybean endosphere bacterial communities, which were further enriched and diversified from week1 to week3/4. Zhalnina et al. (2018) study demonstrated that aromatic compounds (including Nicotinic acid, Cinnamic acid, IAA, Shikimic acid, inositol, vanillin, vanillic acid, salicylic acid) were increasingly released along Avena barbata development, and these increased release of aromatic acid into rhizosphere correlated with the enrichment of bacterial isolates that preferentially utilized aromatic compounds (Zhalnina et al. 2018). The diversified and increased aromatic compound degradation activity within the soybean root bacterial community may reflect the metabolic dynamics within soybean roots, which may change in response to the infection by *Bradyrhizobium japonicum*. As an example, soybean flavonoids and isoflavonoids increased significantly within hairy roots in response to the infection of rhizobia *Brayrhizobium japonicum* (Brechenmacher et al. 2010). Accordingly, Arabidopsis were revealed to release more phenolic (aromatic compounds characterized with a benzene ring) related compounds at the later stages of development (Chaparro et al. 2014; Bhattacharya et al. 2010). The (iso)flavonoids as one major type of phenolic acid have widely studied in terms of its function involved in legume-rhizobia symbiotic interactions. However, other phenolic acid compounds (e.g., vanillin, gallate, p-coumaric acid, and other phenylpropanoids) were revealed to be important signaling molecules mediating plant-microbe interactions and plant pathogen resistance (Mandal et al. 2010; Bhattacharya et al. 2010; Lanoue et al. 2010). Consequently, the dynamic changes of soybean root associated bacteria functions could be attributed to the metabolic changes within soybean root tissue in response to either the pre-programmed development or *Bradyrhizobium japonicum* colonization. To further our understanding, more systemic studies by linking soybean root metabolic profile changes with associated rhizosphere and root microbiome dynamics with both normal soybean and non-nodulating mutant could be the next step of the investigation.

4.4.6 Soybean root fungal community composition and plant compartment impact

Sordariomycetes class predominated in both soil and soybean root associated fungal community across all compartments, followed by *Dothideomycetes*, *Mortierellomycetes*, *Agaricomycetes*, and *Eurotiomycetes*. This fungal community composition is consistent with a former soybean field study based on ITS1 amplicon sequencing (Han et al. 2017). However, soybean root associated fungal composition is less comparable with that reported for wheat, sugarcane, Arabidopsis, poplar, and other woody tree plants (Cregger et al. 2018; Coleman-Derr et al. 2016; Gdanetz and Trail 2017; Bonito et al. 2014). This between species difference could reflect the importance of plant genetic information in mediating general or specific fungi-root interactions. In this study, we found that the compartment-specific recruitment on associated fungi community was less evident in comparison to that on bacterial community, which may relate to the oligotrophic features of fungi that making fungi, in general, less responsive to the increased labile carbon resource of rhizodeposits (Ho et al. 2017). A similar pattern was found for the *Agave* root microbiome, which revealed a strong compartment impact on bacterial community, while the fungal community were more influenced by the biogeography of investigated plant species (Coleman-Derr et al. 2016).

During the early seedling development stage, the *Chaetomium* genus was consistently enriched in the soybean rhizosphere, while Fusarium Solani was selectively enriched in the soybean endosphere. Chaetomium globosum was revealed with a broad spectrum of pathogen suppression activity tested among several crop species, including wheat, rice, and soybean (Aggarwall et al. 2004; Liu et al. 2008; Park et al. 2005; Dhingra et al. 2003). Additionally, Chaetomium globosum NK102 was revealed with nematicidal activity against the root-knot nematode (*Medoidogyne incognita*), which is a damaging pathogen of soybean throughout the southern United States (Hu et al. 2013; Kirkpatrick and May 1989). Although the ITS2 amplicon sequencing-based results were not able to navigate us to species level, the consistent dominance of Chaetomium within the soybean rhizosphere may reflect some intrinsic needs of soybean during early seedling development.

4.4.7 Fungal taxa dynamics along with soybean development

Fusarium solani is a widespread soil-borne fungal pathogen causing soybean sudden death syndrome disease, which could cause yield loss for severely affected areas (Rupe 1989). In this field study, we found that the relative abundance of *Fusarium solani* species was significantly high within the soybean endosphere one week after the soybean germination. In contrast, the abundance of *Fusarium solani* species was comparatively low in the soybean rhizosphere as well as within soybean seeds. This high abundance within soybean roots could be attributed to the quick colonization by surrounding rhizosphere individuals. Alternatively, it could be attributed to seed-derived fungi species, which rapidly multiplied in response to seedling development.

It has been widely recognized that *Actinomycetes* (specifically *Streptomyces*) could produce and secrete a diverse array of biologically active compounds including antibiotics, hydrolytic enzymes and enzyme inhibitors, which equipped *Streptomycetes* with a wide spectrum of antifungal activity against soil-borne pathogens (Anitha and Rabeeth 2010). In fact, *Streptomycetes* species were reported with biocontrol function against *Fusarium solani* across couple crops, including sugarbeet, ginseng, and Chickpea (Moussa and Rizk 2002; Chung et al. 1989; Soltanzadeh et al. 2016). Similarly, *Bacillus* was another PGPR widely reported with biocontrol capacity against *Fusarium solani* (Sallam et al. 2013). Coincidentally, the relative abundance of soybean root bacterial taxa of *Streptomyces* and *Bacillus* genus were actually strikingly high at week1. This may indicate that the beneficial bacteria taxa within soybean roots provided the host with antagonism to *Fusarium solani*. Actually, the dominance of Fusarium solani indeed gradually decreased within soybean roots along with seedling development. Consequently, the reduction of *Fusarium solani* within soybean roots could be a result of the antagonistic interaction between beneficial bacteria/fungi and this pathogen species.

Glomeramycota as AMF fungi was significantly enriched in the soybean rhizosphere at week4 instead of week1/3. Contrarily, within the soybean endosphere, the relative abundance of Glomeromycota was significantly decreased from week1 to week3/4. It has been widely recognized that the symbiotic interaction between plant and AMF could greatly enhance plant drought tolerance, increased available P for plant uptake and stabilizing soil aggregates (Gianinazzi et al. 2010). The high abundance of AMF within soybean at week1 may be a pre-programmed soybean development strategy for seedling establishment. Intriguingly, it has been revealed a prior symbiosis with AMF could greatly facilitate nodule formation considering the high P demand for nodule formation (Meena et al. 2018). Additionally, it has been reported that AMF symbiosis could help to alleviate the premature nodule senescence under drought stress (Ruiz-Lozano et al. 2001). Once soybean seedling was well established, soybean may invest more photosynthetic products in nodule formation instead of AMF symbiosis. Alternatively, it could attribute to the expanded extra-root hypha in the soybean rhizosphere instead of intro-root expansions as reflected by the high AMF fungi abundance in the soybean rhizosphere at week4, which warranted for further investigation.

4.5 Conclusions and perspectives

Our field-based characterization of soybean seed and root associated microbiome along the early development of soybean seedlings illustrated not only a compartment specific structuring but also a development specific recruitment of associate microbial community. Comprehensive characterization and comparison of seed and root microbiome demonstrated that seed carried dominant bacteria was dramatically diluted/competed out by soil-derived microbes even one week after germination. Meantime, we still observed some degree of seed microbiome/ spermosphere legacy effect during the development of root bacteria, which indicated the necessity for a more detailed investigation of the development of root microbiome with a purpose to link/track the trajectory of seed microbiome destiny during the early seedling development stage. Our functional prediction results also pinpointed that the dramatic changes of bacterial metabolic activity in response to soybean development potentially related to the symbiotic interaction between soybean and nodule formation Bradyrhizobium japonicum, which warranted for more comprehensive understanding via coupling root metabolomics with root microbiome dynamics. Fungicide seed treatment as a widely applied modern agriculture practice has brought more attention in terms of their non-target effects on other plant-associated organisms. Our one season-based investigation indicated an insignificant non-target impact on associated bacterial community, while, significant but minor impacts on fungal community overall, with one potentially beneficial biocontrol fungi (Chaetomium) being reduced by fungicide treatment. In fact, long term usage of fungicide may cause more tractable/detectable impacts on non-target organisms, which need more detailed investigation.

This study expanded our understanding of root microbiome assembly by considering of seed microbiome in parallel with soil indigenous microbes. Dynamic assembly of root microbiome highlighted the importance of temporal sampling when studying plant and microbe interaction, especially when considering apply microbe-based products to improve plant performance. Soybean as a legume crop is unique in terms of its root microbiome assembly, a detailed understanding of the interactions between rhizobia and other partners may help to improve soybean nodulation especially under biotic/abiotic stress environments.

Reference

Aggarwall, Rashmi, A. K. Tewari, K. D. Srivastava, and D. V. Singh. 2004. “Role of Antibiosis in the Biological Control of Spot Blotch (Cochliobolus Sativus) of Wheat by Chaetomium Globosum.” Mycopathologia 157 (4): 369–77.

Almario, Juliana, Ganga Jeena, Jörg Wunder, Gregor Langen, Alga Zuccaro, George Coupland, and Marcel Bucher. 2017. “Root-Associated Fungal Microbiota of Nonmycorrhizal Arabis Alpina and Its Contribution to Plant Phosphorus Nutrition.” Proceedings of the National Academy of Sciences of the United States of America 114 (44): E9403–12.

Anitha, A., and M. Rabeeth. 2010. “Degradation of Fungal Cell Walls of Phytopathogenic Fungi by Lytic Enzyme of Streptomyces Griseus.” African Journal of Plant Science 4 (3): 061–066.

Bais, Harsh P., Tiffany L. Weir, Laura G. Perry, Simon Gilroy, and Jorge M. Vivanco. 2006. “The role of root exudates in rhizosphere interactions with plants and other organisms.” Annual Review of Plant Biology 57: 233–66.

Bakker, Peter A. H. M., Corné M. J. Pieterse, Ronnie de Jonge, and Roeland L. Berendsen. 2018. “The Soil-Borne Legacy.” Cell 172 (6): 1178–80.

Barret, Matthieu, Martial Briand, Sophie Bonneau, Anne Préveaux, Sophie Valière, Olivier Bouchez, Gilles Hunault, Philippe Simoneau, and Marie-Agnès Jacquesa. 2015. “Emergence Shapes the Structure of the Seed Microbiota.” Applied and Environmental Microbiology 81 (4): 1257–66.

Beattie, Gwyn A. 2018. “Metabolic Coupling on Roots.” Nature Microbiology 3 (4): 396.

Berg, Gabriele, Martin Grube, Michael Schloter, and Kornelia Smalla. 2014. “Unraveling the Plant Microbiome: Looking Back and Future Perspectives.” Frontiers in Microbiology 5 (June): 148.

Bewley, J. Derek. 1997. “Seed Germination and Dormancy.” The Plant Cell 9 (7): 1055.

Bhattacharya, Amita, Priyanka Sood, and Vitaly Citovsky. 2010. “The Roles of Plant Phenolics in Defence and Communication during Agrobacterium and Rhizobium Infection.” Molecular Plant Pathology 11 (5): 705–19.

Bonito, Gregory, Hannah Reynolds, Michael S. Robeson, Jessica Nelson, Brendan P. Hodkinson, Gerald Tuskan, Christopher W. Schadt, and Rytas Vilgalys. 2014. “Plant Host and Soil Origin Influence Fungal and Bacterial Assemblages in the Roots of Woody Plants.” Molecular Ecology 23 (13): 3356–70.

Brechenmacher, Laurent, Zhentian Lei, Marc Libault, Seth Findley, Masayuki Sugawara, Michael J. Sadowsky, Lloyd W. Sumner, and Gary Stacey. 2010. “Soybean Metabolites Regulated in Root Hairs in Response to the Symbiotic Bacterium Bradyrhizobium Japonicum.” Plant Physiology 153 (4): 1808–22.

Bulgarelli, Davide, Matthias Rott, Klaus Schlaeppi, Emiel Ver Loren van Themaat, Nahal Ahmadinejad, Federica Assenza, Philipp Rauf, et al. 2012. “Revealing Structure and Assembly Cues for Arabidopsis Root-Inhabiting Bacterial Microbiota.” Nature 488 (7409): 91–95.

Bulgarelli, Davide, Klaus Schlaeppi, Stijn Spaepen, Emiel Ver Loren van Themaat, and Paul Schulze-Lefert. 2013. “Structure and Functions of the Bacterial Microbiota of Plants.” Annual Review of Plant Biology 64 (January): 807–38.

Buysens, Catherine, Hervé Dupré de Boulois, and Stéphane Declerck. 2015. “Do Fungicides Used to Control Rhizoctonia Solani Impact the Non-Target Arbuscular

Mycorrhizal Fungus Rhizophagus Irregularis?” Mycorrhiza 25 (4): 277–88.

Cameron, Jesse C., R. Michael Lehman, Peter Sexton, Shannon L. Osborne, and Wendy I. Taheri. 2017. “Fungicidal Seed Coatings Exert Minor Effects on Arbuscular Mycorrhizal Fungi and Plant Nutrient Content.” Agronomy Journal 109: 1005–12.

Chaparro, Jacqueline M., Dayakar V. Badri, Matthew G. Bakker, Akifumi Sugiyama, Daniel K. Manter, and Jorge M. Vivanco. 2013. “Root Exudation of Phytochemicals in Arabidopsis Follows Specific Patterns That Are Developmentally Programmed and Correlate with Soil Microbial Functions.” PLoS ONE 8 (2): e55731.

Chaparro, Jacqueline M., Dayakar V. Badri, and Jorge M. Vivanco. 2014. “Rhizosphere Microbiome Assemblage Is Affected by Plant Development.” The ISME Journal 8 (4): 790–803.

Chen, Haimin, Hongxia Wu, Bin Yan, Hongguang Zhao, Fenghua Liu, Haihua Zhang, Qing Sheng, Fang Miao, and Zongsuo Liang. 2018. “Core Microbiome of Medicinal Plant Salvia Miltiorrhiza Seed: A Rich Reservoir of Beneficial Microbes for Secondary Metabolism?” International Journal of Molecular Sciences 19 (3): 672.

Chung, Y. R., Ohh, S. H., & Chung, H. S. (1989). “Antagonistic activity of Streptomyces species against Fusarium solani causing ginseng root rot.” The Korean Journal of Microbiology (Korea R.).

Coleman-Derr, Devin, Damaris Desgarennes, Citlali Fonseca-Garcia, Stephen Gross, Scott Clingenpeel, Tanja Woyke, Gretchen North, Axel Visel, Laila P. Partida-Martinez, and Susannah G. Tringe. 2016. “Plant Compartment and Biogeography Affect Microbiome Composition in Cultivated and Native Agave Species.” The New

Phytologist 209 (2): 798–811.

Cregger, M. A., A. M. Veach, Z. K. Yang, M. J. Crouch, R. Vilgalys, G. A. Tuskan, and C. W. Schadt. 2018. “The Populus Holobiont: Dissecting the Effects of Plant Niches and Genotype on the Microbiome.” Microbiome 6 (1): 31.

Dhingra, O. D., E. S. G. Mizubuti, and F. M. Santana. 2003. “Chaetomium Globosum for Reducing Primary Inoculum of Diaporthe Phaseolorum F. Sp. Meridionalis in Soil-Surface Soybean Stubble in Field Conditions.” Biological Control: Theory and Applications in Pest Management 26 (3): 302–10.

Dijk, K. van, and E. B. Nelson. 2000. “Fatty Acid Competition as a Mechanism by Which Enterobacter Cloacae Suppresses Pythium Ultimum Sporangium Germination and Damping-Off.” Applied and Environmental Microbiology 66 (12): 5340–47.

Douglas, Gavin M., Vincent J. Maffei, Jesse Zaneveld, Svetlana N. Yurgel, James R. Brown, Christopher M. Taylor, Curtis Huttenhower, and Morgan G. I. Langille. 2019. “PICRUSt2: An Improved and Extensible Approach for Metagenome Inference.” bioRxiv: 672295.

Edwards, Joseph A., Christian M. Santos-Medellín, Zachary S. Liechty, Bao Nguyen, Eugene Lurie, Shane Eason, Gregory Phillips, and Venkatesan Sundaresan. 2018. “Compositional Shifts in Root-Associated Bacterial and Archaeal Microbiota Track the Plant Life Cycle in Field-Grown Rice.” PLoS Biology 16 (2): e2003862.

Edwards, Joseph, Cameron Johnson, Christian Santos-Medellín, Eugene Lurie, Natraj Kumar Podishetty, Srijak Bhatnagar, Jonathan A. Eisen, and Venkatesan Sundaresan. 2015. “Structure, Variation, and Assembly of the Root-Associated Microbiomes of Rice.” Proceedings of the National Academy of Sciences of the

United States of America 112 (8): E911–20.

Galbraith, M. P., S. F. Feng, J. Borneman, E. W. Triplett, F. J. de Bruijn, and S. Rossbach. 1998. “A Functional Myo-Inositol Catabolism Pathway Is Essential for Rhizopine Utilization by Sinorhizobium Meliloti.” Microbiology 144 (10) (October): 2915–24.

Gdanetz, Kristi, and Frances Trail. 2017. “The Wheat Microbiome Under Four Management Strategies, and Potential for Endophytes in Disease Protection.” Phytobiomes Journal 1 (3): 158–68.

Gianinazzi, Silvio, Armelle Gollotte, Marie-Noëlle Binet, Diederik van Tuinen, Dirk Redecker, and Daniel Wipf. 2010. “Agroecology: The Key Role of Arbuscular Mycorrhizas in Ecosystem Services.” Mycorrhiza 20 (8): 519–30.

Goh, Chooi-Hua, Debora F. Veliz Vallejos, Adrienne B. Nicotra, and Ulrike Mathesius. 2013. “The Impact of Beneficial Plant-Associated Microbes on Plant Phenotypic Plasticity.” Journal of Chemical Ecology 39 (7): 826–39.

Gruber, Benjamin D., Ricardo F. H. Giehl, Swetlana Friedel, and Nicolaus von Wirén. 2013. “Plasticity of the Arabidopsis Root System under Nutrient Deficiencies.” Plant Physiology 163 (1): 161–79.

Han, Li-Li, Jun-Tao Wang, Sheng-Hui Yang, Wen-Feng Chen, Li-Mei Zhang, and Ji-Zheng He. 2017. “Temporal Dynamics of Fungal Communities in Soybean Rhizosphere.” Journal of Soils and Sediments 17 (2): 491–98.

Hartmann, Anton, Michael Rothballer, and Michael Schmid. 2008. “Lorenz Hiltner, a Pioneer in Rhizosphere Microbial Ecology and Soil Bacteriology Research.” Plant and Soil 312 (1): 7–14.

Hiltner, Lorenz 1904. “Uber Nevere Erfahrungen Und Probleme Auf Dem Gebiet Der Boden Bakteriologie Und Unter Besonderer Beurchsichtigung Der Grundungung Und Broche.” Arbeit. Deut. Landw. Ges. Berlin 98: 59–78.

Ho, Adrian, D. Paolo Di Lonardo, and Paul L. E. Bodelier. 2017. “Revisiting Life Strategy Concepts in Environmental Microbial Ecology.” FEMS Microbiology Ecology 93 (3): fix006.

Htwe, Aung Zaw, Seinn Moh Moh, Khin Myat Soe, Kyi Moe, and Takeo Yamakawa. 2019. “Effects of Biofertilizer Produced from Bradyrhizobium and Streptomyces Griseoflavus on Plant Growth, Nodulation, Nitrogen Fixation, Nutrient Uptake, and Seed Yield of Mung Bean, Cowpea, and Soybean.” Agronomy 9 (2): 77.

Huang, Ancheng C., Ting Jiang, Yong-Xin Liu, Yue-Chen Bai, James Reed, Baoyuan Qu, Alain Goossens, Hans-Wilhelm Nützmann, Yang Bai, and Anne Osbourn. 2019. “A Specialized Metabolic Network Selectively Modulates Arabidopsis Root Microbiota.” Science 364 (6440): eaau6389.

Huang, Yali, Zaoyuan Kuang, Wenfeng Wang, and Lixiang Cao. 2016. “Exploring Potential Bacterial and Fungal Biocontrol Agents Transmitted from Seeds to Sprouts of Wheat.” Biological Control: Theory and Applications in Pest Management 98 (July): 27–33.

Hu, Yang, Weipu Zhang, Ping Zhang, Weibin Ruan, and Xudong Zhu. 2013. “Nematicidal Activity of Chaetoglobosin A Poduced by Chaetomium Globosum NK102 against Meloidogyne Incognita.” Journal of Agricultural and Food Chemistry 61 (1): 41–46.

Jez, Joseph M., Soon Goo Lee, and Ashley M. Sherp. 2016. “The next Green

Movement: Plant Biology for the Environment and Sustainability.” Science 353 (6305): 1241–44.

Jiang, G., A. H. Krishnan, Y. W. Kim, T. J. Wacek, and H. B. Krishnan. 2001. “A Functional Myo-Inositol Dehydrogenase Gene Is Required for Efficient Nitrogen Fixation and Competitiveness of Sinorhizobium Fredii USDA191 to Nodulate Soybean (Glycine Max [L.] Merr.).” Journal of Bacteriology 183 (8): 2595–2604.

Jin, Hongyan, James J. Germida, and Fran L. Walley. 2013. “Suppressive Effects of Seed-Applied Fungicides on Arbuscular Mycorrhizal Fungi (AMF) Differ with Fungicide Mode of Action and AMF Species.” Applied Soil Ecology: A Section of Agriculture, Ecosystems & Environment 72 (October): 22–30.

Jog, R., M. Pandya, G. Nareshkumar, and S. Rajkumar. 2014. “Mechanism of Phosphate Solubilization and Antifungal Activity of Streptomyces Spp. Isolated from Wheat Roots and Rhizosphere and Their Application in Improving Plant Growth.” Microbiology 160 (4): 778–88.

Johnston-Monje, David, Derek S. Lundberg, George Lazarovits, Veronica M. Reis, and Manish N. Raizada. 2016. “Bacterial Populations in Juvenile Maize Rhizospheres Originate from Both Seed and Soil.” Plant and Soil 405 (1): 337–55.

Kageyama, Koji, and Eric B. Nelson. 2003. “Differential Inactivation of Seed Exudate Stimulation of Pythium Ultimum Sporangium Germination by Enterobacter Cloacae Influences Biological Control Efficacy on Different Plant Species.” Applied and Environmental Microbiology 69 (2): 1114–20.

Kirkpatrick, T. L., and M. L. May. 1989. “Host Suitability of Soybean Cultivars for Meloidogyne Incognita and M. Arenaria.” Journal of Nematology 21 (4): 666–70.

Klindworth, Anna, Elmar Pruesse, Timmy Schweer, Jörg Peplies, Christian Quast, Matthias Horn, and Frank Oliver Glöckner. 2013. “Evaluation of General 16S Ribosomal RNA Gene PCR Primers for Classical and next-Generation Sequencing-Based Diversity Studies.” Nucleic Acids Research 41 (1): e1.

Kozich, James J., Sarah L. Westcott, Nielson T. Baxter, Sarah K. Highlander, and Patrick D. Schloss. 2013. “Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform.” Applied and Environmental Microbiology 79 (17): 5112–20.

Kyei-Boahen, S., A. E. Slinkard, and F. L. Walley. 2001. “Rhizobial Survival and Nodulation of Chickpea as Influenced by Fungicide Seed Treatment.” Canadian Journal of Microbiology 47 (6): 585–89.

Lanoue, Arnaud, Vincent Burlat, Ulrich Schurr, and Ursula S. R. Röse. 2010. “Induced Root-Secreted Phenolic Compounds as a Belowground Plant Defense.” Plant Signaling & Behavior 5 (8): 1037–38.

Lebeis, Sarah L., Sur Herrera Paredes, Derek S. Lundberg, Natalie Breakfield, Jase Gehring, Meredith McDonald, Stephanie Malfatti, et al. 2015. “Salicylic Acid Modulates Colonization of the Root Microbiome by Specific Bacterial Taxa.” Science 349 (6250): 860–64.

Levy, Asaf, Jonathan M. Conway, Jeffery L. Dangl, and Tanja Woyke. 2018. “Elucidating Bacterial Gene Functions in the Plant Microbiome.” Cell Host & Microbe 24 (4): 475–85.

Levy, Asaf, Isai Salas Gonzalez, Maximilian Mittelviefhaus, Scott Clingenpeel, Sur Herrera Paredes, Jiamin Miao, Kunru Wang, et al. 2018. “Genomic Features of

Bacterial Adaptation to Plants.” Nature Genetics 50 (1): 138–50.

Li, Huashan, Peng Lei, Xiao Pang, Sha Li, Hong Xu, Zongqi Xu, and Xiaohai Feng. 2017. “Enhanced Tolerance to Salt Stress in Canola (Brassica Napus L.) Seedlings Inoculated with the Halotolerant Enterobacter Cloacae HSNJ4.” Applied Soil Ecology: A Section of Agriculture, Ecosystems & Environment 119 (October): 26–34.

Liu, Fang, Tarek Hewezi, Sarah L. Lebeis, Vince Pantalone, Parwinder S. Grewal, and Margaret E. Staton. 2019. “Soil Indigenous Microbiome and Plant Genotypes Cooperatively Modify Soybean Rhizosphere Microbiome Assembly.” BMC Microbiology 19 (1): 201.

Liu, Yang, Shan Zuo, Yuanyuan Zou, Jianhua Wang, and Wei Song. 2012. “Investigation on Diversity and Population Succession Dynamics of Indigenous Bacteria of the Maize Spermosphere.” World Journal of Microbiology and Biotechnology 28 (1): 391–6.

Liu, Z. H., Q. Yang, S. Hu, J. D. Zhang, and J. Ma. 2008. “Cloning and Characterization of a Novel Chitinase Gene (chi46) from Chaetomium Globosum and Identification of Its Biological Activity.” Applied Microbiology and Biotechnology 80 (2): 241–52.

Lundberg, Derek S., Sarah L. Lebeis, Sur Herrera Paredes, Scott Yourstone, Jase Gehring, Stephanie Malfatti, Julien Tremblay, et al. 2012. “Defining the Core Arabidopsis Thaliana Root Microbiome.” Nature 488 (7409): 86–90.

Lundberg, Derek S., Scott Yourstone, Piotr Mieczkowski, Corbin D. Jones, and Jeffery L. Dangl. 2013. “Practical Innovations for High-Throughput Amplicon Sequencing.” Nature Methods 10 (10): 999–1002.

Majumdar, S. K., and H. J. Kutzner. 1962. “Myo-Inositol in the Biosynthesis of Streptomycin by Streptomyces Griseus.” Science 135 (3505): 734.

Mandal, Santi M., Dipjyoti Chakraborty, and Satyahari Dey. 2010. “Phenolic Acids Act as Signaling Molecules in Plant-Microbe Symbioses.” Plant Signaling & Behavior 5 (4): 359–68.

Mano, Hironobu, Fumiko Tanaka, Asuka Watanabe, Hiroko Kaga, Suguru Okunishi, and Hisao Morisaki. 2006. “Culturable Surface and Endophytic Bacterial Flora of the Maturing Seeds of Rice Plants (Oryza Sativa) Cultivated in a Paddy Field.” Microbes and Environments JSME 21 (2): 86–100.

Martinez-Rodriguez, America, Gloria Macedo-Raygoza, Aurora X. Huerta-Robles, Ileana Reyes-Sepulveda, Jhovana Lozano-Lopez, Evelyn Y. García-Ochoa, Luis Fierro-Kong, et al. 2019. “Agave Seed Endophytes: Ecology and Impacts on Root Architecture, Nutrient Acquisition, and Cold Stress Tolerance.” In Seed Endophytes: Biology and Biotechnology, edited by Satish Kumar Verma, White, Jr, and James Francis, 139–70. Cham: Springer International Publishing.

McInerney, Michael J., Lars Rohlin, Housna Mouttaki, Unmi Kim, Rebecca S. Krupp, Luis Rios-Hernandez, Jessica Sieber, et al. 2007. “The Genome of Syntrophus Aciditrophicus: Life at the Thermodynamic Limit of Microbial Growth.” Proceedings of the National Academy of Sciences of the United States of America 104 (18): 7600–7605.

McInerney, Michael J., Jessica R. Sieber, and Robert P. Gunsalus. 2009. “Syntrophy in Anaerobic Global Carbon Cycles.” Current Opinion in Biotechnology 20 (6): 623–32.

Meena, Ram Swaroop, Vinod Vijayakumar, Gulab Singh Yadav, and Tarik Mitran. 2018.

“Response and Interaction of Bradyrhizobium Japonicum and Arbuscular Mycorrhizal Fungi in the Soybean Rhizosphere.” Plant Growth Regulation 84 (2): 207–23.

Mendes, Rodrigo, Marco Kruijt, Irene de Bruijn, Ester Dekkers, Menno van der Voort, Johannes H. M. Schneider, Yvette M. Piceno, et al. 2011. “Deciphering the Rhizosphere Microbiome for Disease-Suppressive Bacteria.” Science 332 (6033): 1097–1100.

Moussa, Tarek A. A., and Mohamed A. Rizk. 2002. “Biocontrol of Sugarbeet Pathogen Fusarium Solani (Mart.) Sacc. by Streptomyces Aureofaciens.” Pakistan Journal of Biological Sciences: PJBS 5 (5): 556–59.

Mubeen, Fathia, Munir A. Shiekh, Tahira Iqbal, Qaiser M. Khan, Kauser A. Malik, and Fauzia Y. Hafeez. 2006. “In Vitro Investigations to Explore the Toxicity of Fungicides for Plant Growth Promoting Rhizobacteria.” Pakistan Journal of Botany 38 (4): 1261.

Müller, Daniel B., Christine Vogel, Yang Bai, and Julia A. Vorholt. 2016. “The Plant Microbiota: Systems-Level Insights and Perspectives.” Annual Review of Genetics 50 (November): 211–34.

Munkvold, Gary P. 2009. “Seed Pathology Progress in Academia and Industry.” Annual Review of Phytopathology 47: 285–311.

Muthomi, J. W., P. E. Otieno, G. N. Chemining’wa, J. H. Nderitu, J. M. Wagacha. 2007. “Effect of Legume Root Rot Pathogens and Fungicide Seed Treatment on Nodulation and Biomass Accumulation.” The Journal of Biological Sciences 7: 1163–70.

Nelson, Eric B. 2004. “Microbial Dynamics and Interactions in the Spermosphere.” Annual Review of Phytopathology 42: 271–309.

Nelson, E.B., 2018. “The Seed Microbiome: Origins, Interactions, and Impacts.” Plant and Soil 422 (1): 7–34.

Nelson, Eric B., Philippe Simoneau, Matthieu Barret, Birgit Mitter, and Stéphane Compant. 2018. “Editorial Special Issue: The Soil, the Seed, the Microbes and the Plant.” Plant and Soil 422 (1): 1–5.

Nilsson, Rolf Henrik, Karl-Henrik Larsson, Andy F. S. Taylor, Johan Bengtsson-Palme, Thomas S. Jeppesen, Dmitry Schigel, Peter Kennedy, et al. 2019. “The UNITE Database for Molecular Identification of Fungi: Handling Dark Taxa and Parallel Taxonomic Classifications.” Nucleic Acids Research 47 (1): D259–64.

Padilla, Francisco M., Juan de Dios Miranda, and Francisco I. Pugnaire. 2007. “Early Root Growth Plasticity in Seedlings of Three Mediterranean Woody Species.” Plant and Soil 296 (1): 103–13.

Palaniyandi, S. A., K. Damodharan, S. H. Yang, and J. W. Suh. 2014. “Streptomyces Sp. Strain PGPA39 Alleviates Salt Stress and Promotes Growth of ‘Micro Tom’ Tomato Plants.” Journal of Applied Microbiology 117 (3): 766–73.

Park, Joong-Hyeop, Gyung Ja Choi, Kyoung Soo Jang, He Kyoung Lim, Heung Tae Kim, Kwang Yun Cho, and Jin-Cheol Kim. 2005. “Antifungal Activity against Plant Pathogenic Fungi of Chaetoviridins Isolated from Chaetomium Globosum.” FEMS Microbiology Letters 252 (2): 309–13.

Philippot, Laurent, Jos M. Raaijmakers, Philippe Lemanceau, and Wim H. van der Putten. 2013. “Going back to the Roots: The Microbial Ecology of the Rhizosphere.”

Nature Reviews. Microbiology 11 (11): 789–99.

Pieterse, Corné M. J., Ronnie de Jonge, and Roeland L. Berendsen. 2016. “The Soil-Borne Supremacy.” Trends in Plant Science 21 (3): 171–73.

Quast, Christian, Elmar Pruesse, Pelin Yilmaz, Jan Gerken, Timmy Schweer, Pablo Yarza, Jörg Peplies, and Frank Oliver Glöckner. 2013. “The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools.” Nucleic Acids Research 41 (Database issue): 590–96.

R Development Core Team, Rffsc, and Others. 2011. “R: A Language and Environment for Statistical Computing.” R foundation for statistical computing Vienna, Austria.

Rezki, Samir, Claire Campion, Philippe Simoneau, Marie-Agnès Jacques, Ashley Shade, and Matthieu Barret. 2018. “Assembly of Seed-Associated Microbial Communities within and across Successive Plant Generations.” Plant and Soil 422 (1): 67–79.

Robbins, Neil E., and José R. Dinneny. 2015. “The Divining Root: Moisture-Driven Responses of Roots at the Micro- and Macro-Scale.” Journal of Experimental Botany 66 (8): 2145–54.

Rognes, Torbjørn, Tomáš Flouri, Ben Nichols, Christopher Quince, and Frédéric Mahé. 2016. “VSEARCH: A Versatile Open Source Tool for Metagenomics.” PeerJ 4 (October): e2584.

Rothrock, C.S., Winters, S.A., Barham, J.D., Hope, A.R., Beach, A.B., Keiser, A.R., Bayles, M.B., Caceres, J., Colyer, P.D. and AgCenter, L.S.U., 2004. “Report of the cottonseed treatment committee for 2003”. 2004 Beltwide Cotton Conf., National Cotton Council, Memphis, TN. CD-ROM.

Ruiza, Dante, Betina Agaras, Patrice de Werrab, Luis G. Wall, and Claudio Valverde. 2011. “Characterization and Screening of Plant Probiotic Traits of Bacteria Isolated from Rice Seeds Cultivated in Argentina.” The Journal of Microbiology 49 (6): 902–12.

Ruiz-Lozano, Juan Manuel, Carlos Collados, Jose Miguel Barea, and Rosario Azcon. 2001. “Arbuscular Mycorrhizal Symbiosis Can Alleviate Drought-Induced Nodule Senescence in Soybean Plants.” New Phytologist 151 (2): 493–502.

Rupe, J. C. 1989. “Frequency and Pathogenicity of Fusarium Solani Recovered from Soybeans with Sudden Death Syndrome.” Plant Disease 581.

Salanoubat, M., S. Genin, F. Artiguenave, J. Gouzy, S. Mangenot, M. Arlat, A. Billault, et al. 2002. “Genome Sequence of the Plant Pathogen Ralstonia Solanacearum.” Nature 415 (6871): 497–502.

Sallam, Nashwa Atef, Shaimaa Nagy Riad, Mohamed Samy Mohamed, and Ahmed Seef El-eslam. 2013. “Formulations of Bacillus Spp. and Pseudomonas Fluorescens for Biocontrol of Cantaloupe Root Rot Caused by Fusarium Solani.” Journal of Plant Protection Research 53 (3): 295–300.

Schiltz, S., I. Gaillard, N. Pawlicki-Jullian, B. Thiombiano, F. Mesnard, and E. Gontier. 2015. “A Review: What Is the Spermosphere and How Can It Be Studied?” Journal of Applied Microbiology 119 (6): 1467–81.

Schlaeppi, Klaus, and Davide Bulgarelli. 2015. “The Plant Microbiome at Work.” Molecular Plant-Microbe Interactions: MPMI 28 (3): 212–17.

Segata, Nicola, Jacques Izard, Levi Waldron, Dirk Gevers, Larisa Miropolsky, Wendy S. Garrett, and Curtis Huttenhower. 2011. “Metagenomic Biomarker Discovery and

Explanation.” Genome Biology 12 (6): R60.

Shade, Ashley, Marie-Agnès Jacques, and Matthieu Barret. 2017. “Ecological Patterns of Seed Microbiome Diversity, Transmission, and Assembly.” Current Opinion in Microbiology 37 (June): 15–22.

Shi, Shengjing, Erin Nuccio, Donald J. Herman, Ruud Rijkers, Katerina Estera, Jiabao Li, Ulisses Nunes da Rocha, et al. 2015. “Successional Trajectories of Rhizosphere Bacterial Communities over Consecutive Seasons.” mBio 6 (4): e00746.

Solorzano, C. D., and D. K. Malvick. 2011. “Effects of Fungicide Seed Treatments on Germination, Population, and Yield of Maize Grown from Seed Infected with Fungal Pathogens.” Field Crops Research 122 (3): 173–78.

Soltanzadeh, Mahboobeh, Meysam Soltani Nejad, and Gholam Hosein Shahidi Bonjar. 2016. “Application of Soil-Borne Actinomycetes for Biological Control against Fusarium Wilt of Chickpea (Cicer Arietinum) Caused by Fusarium Solani Fsp Pisi.” Journal of Phytopathology 164 (11-12): 967–978.

Soytong, K., S. Kanokmedhakul, V. Kukongviriyapa, and M. Isobe. 2001. “Application of Chaetomium Species (Ketomium) as a New Broad Spectrum Biological Fungicide for Plant Disease Control.” Fungal Diversity 7: 1–15.

Stringlis, Ioannis A., Ke Yu, Kirstin Feussner, Ronnie de Jonge, Sietske Van Bentum, Marcel C. Van Verk, Roeland L. Berendsen, Peter A. H. M. Bakker, Ivo Feussner, and Corné M. J. Pieterse. 2018. “MYB72-Dependent Coumarin Exudation Shapes Root Microbiome Assembly to Promote Plant Health.” Proceedings of the National Academy of Sciences of the United States of America 115 (22): E5213–22.

Subramanian, Parthiban, Kiyoon Kim, Ramasamy Krishnamoorthy, Subbiah Sundaram, and Tongmin Sa. 2015. “Endophytic Bacteria Improve Nodule Function and Plant Nitrogen in Soybean on Co-Inoculation with Bradyrhizobium Japonicum MN110.” Plant Growth Regulation 76 (3): 327–32.

Truyens, S., N. Weyens, and A. Cuypers. 2015. “Bacterial Seed Endophytes: Genera, Vertical Transmission and Interaction with Plants.” The Environmentalist 7 (1): 40–50.

Tveit, M., and R. K. S. Wood. 1955. “The control of Fusarium blight in oat seedlings with antagonistic species of Chaetomium.” The Annals of Applied Biology 43 (4): 538–52.

Urrea, K., J. C. Rupe, and C. S. Rothrock. 2013. “Effect of Fungicide Seed Treatments, Cultivars, and Soils on Soybean Stand Establishment.” Plant Disease 97 (6): 807–12.

Vandenkoornhuyse, Philippe, Achim Quaiser, Marie Duhamel, Amandine Le Van, and Alexis Dufresne. 2015. “The Importance of the Microbiome of the Plant Holobiont.” The New Phytologist 206 (4): 1196–1206.

Vasconcellos, Rafael Leandro Figueiredo de, and Elke Jurandy Bran Nogueira Cardoso. 2009. “Rhizospheric Streptomycetes as Potential Biocontrol Agents of Fusarium and Armillaria Pine Rot and as PGPR for Pinus Taeda.” Biocontrol 54 (6): 807.

Weems, J. D., J. S. Haudenshield, J. P. Bond, G. L. Hartman, K. A. Ames, and C. A. Bradley. 2015. “Effect of Fungicide Seed Treatments on Fusarium Virguliforme Infection of Soybean and Development of Sudden Death Syndrome.” Canadian Journal of Plant Pathology. Revue Canadienne de Phytopathologie 37 (4): 435–47.

Weller, David M. 2007. “Pseudomonas Biocontrol Agents of Soilborne Pathogens: Looking Back over 30 Years.” Phytopathology 97 (2): 250–56.

Western, Tamara L. 2012. “The Sticky Tale of Seed Coat Mucilages: Production, Genetics, and Role in Seed Germination and Dispersal.” Seed Science Research 22 (1): 1–25.

Windstam, Sofia, and Eric B. Nelson. 2008. “Differential Interference with Pythium Ultimum Sporangial Activation and Germination by Enterobacter Cloacae in the Corn and Cucumber Spermospheres.” Applied and Environmental Microbiology 74 (14): 4285–91.

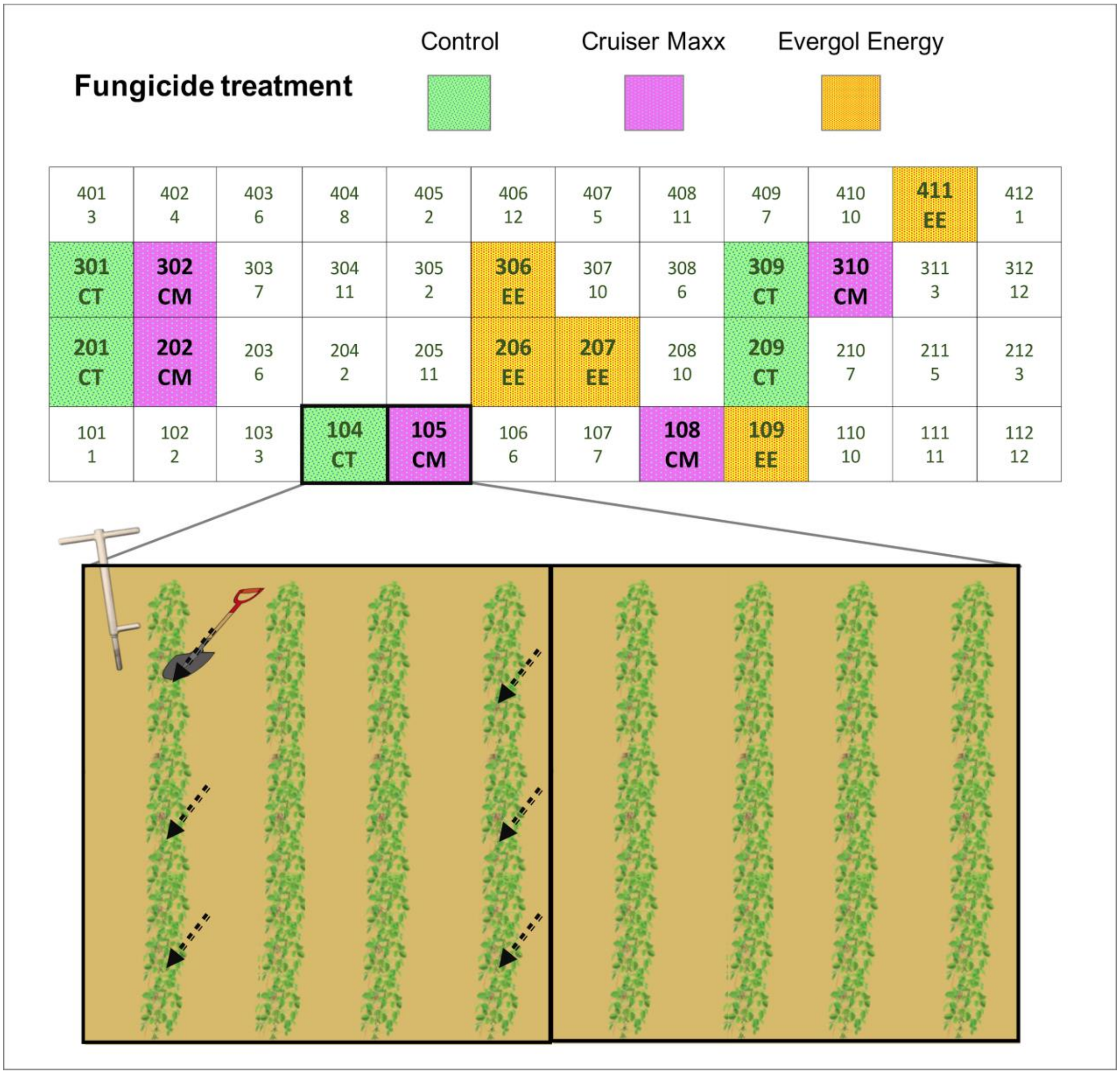
Xiao, Xiao, Weimin Chen, Le Zong, Jun Yang, Shuo Jiao, Yanbing Lin, Entao Wang, and Gehong Wei. 2017. “Two Cultivated Legume Plants Reveal the Enrichment Process of the Microbiome in the Rhizocompartments.” Molecular Ecology 26 (6): 1641–51.

Xu, Ling, Dan Naylor, Zhaobin Dong, Tuesday Simmons, Grady Pierroz, Kim K. Hixson, Young-Mo Kim, et al. 2018. “Drought Delays Development of the Sorghum Root Microbiome and Enriches for Monoderm Bacteria.” Proceedings of the National Academy of Sciences of the United States of America 115 (18): E4284–93.

Yang, Luhua, Jasmin Danzberger, Anne Schöler, Peter Schröder, Michael Schloter, and Viviane Radl. 2017. “Dominant Groups of Potentially Active Bacteria Shared by Barley Seeds Become Less Abundant in Root Associated Microbiome.” Frontiers in Plant Science 8 (June): 1005.

Zhalnina, Kateryna, Katherine B. Louie, Zhao Hao, Nasim Mansoori, Ulisses Nunes da Rocha, Shengjing Shi, Heejung Cho, et al. 2018. “Dynamic Root Exudate Chemistry and Microbial Substrate Preferences Drive Patterns in Rhizosphere Microbial Community Assembly." Nature Microbiology 3 (4): 470–80.

Appendices 4



Fungicide treatment

Control 🟩

Cruiser Maxx 🟪

Evergol Energy 🟧

401 3	402 4	403 6	404 8	405 2	406 12	407 5	408 11	409 7	410 10	**411** **EE**	412 1
301 **CT**	**302** **CM**	303 7	304 11	305 2	**306** **EE**	307 10	308 6	**309** **CT**	**310** **CM**	311 3	312 12
201 **CT**	**202** **CM**	203 6	204 2	205 11	**206** **EE**	**207** **EE**	208 10	**209** **CT**	210 7	211 5	212 3
101 1	102 2	103 3	**104** **CT**	**105** **CM**	106 6	107 7	**108** **CM**	**109** **EE**	110 10	111 11	112 12

Fig. 4.1 Plot design for fungicide seed treatment and sample collection strategy.

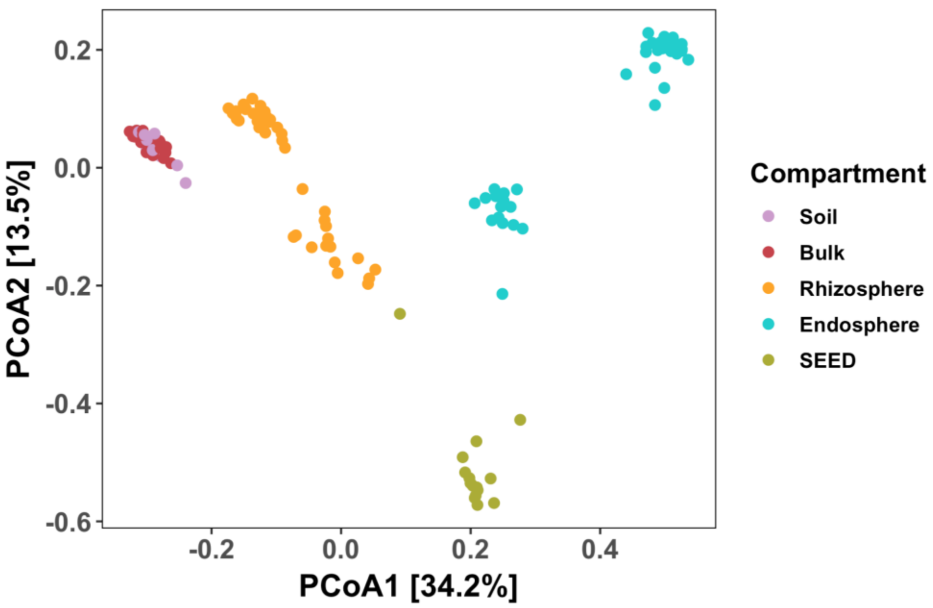


Fig. 4.2 Bacteria composition between compartments.

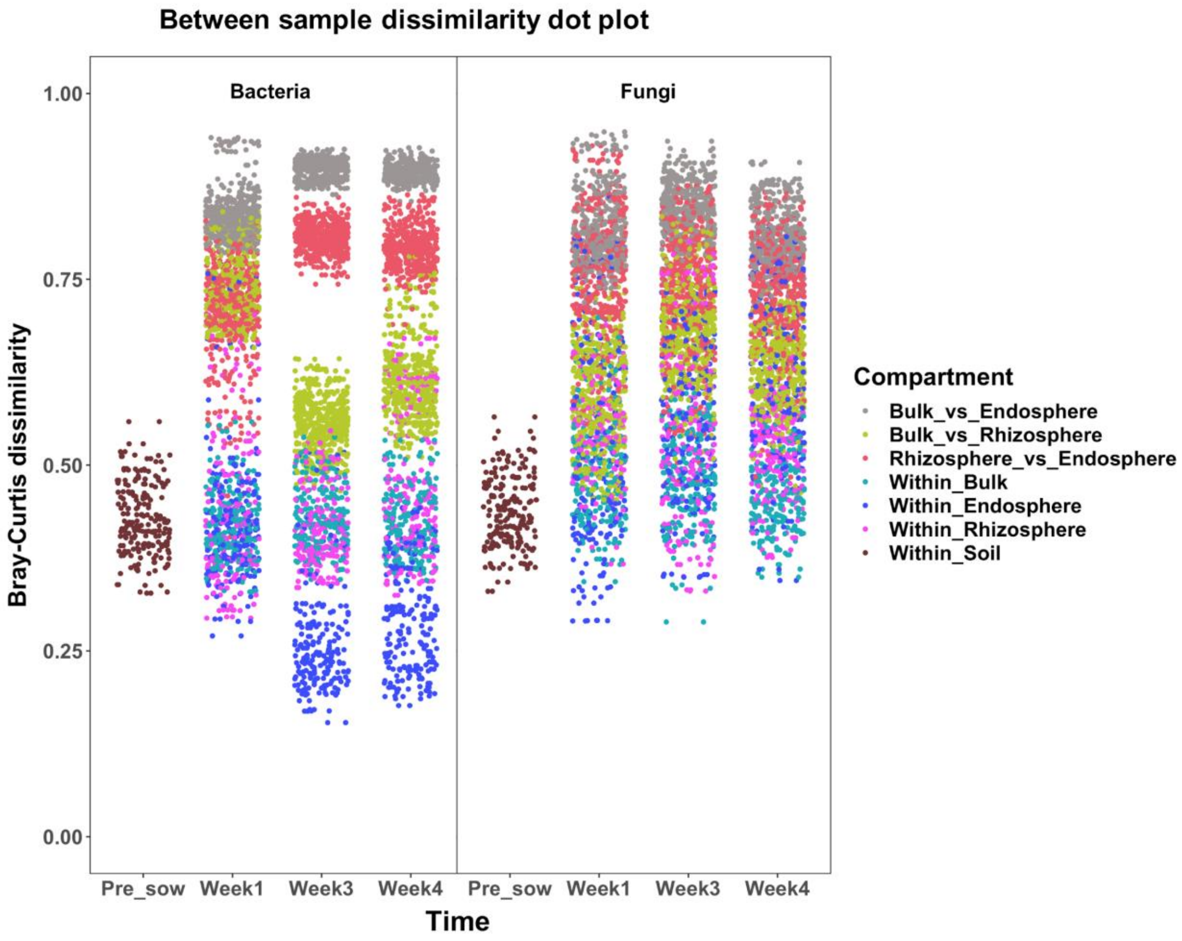


Fig. 4.3 Bray-Curtis dissimilarity between compartments along with soybean development.

The beta diversity between each pair of samples were indicated by Bray-Curtis dissimilarity, the larger the value the more different the community between samples were.

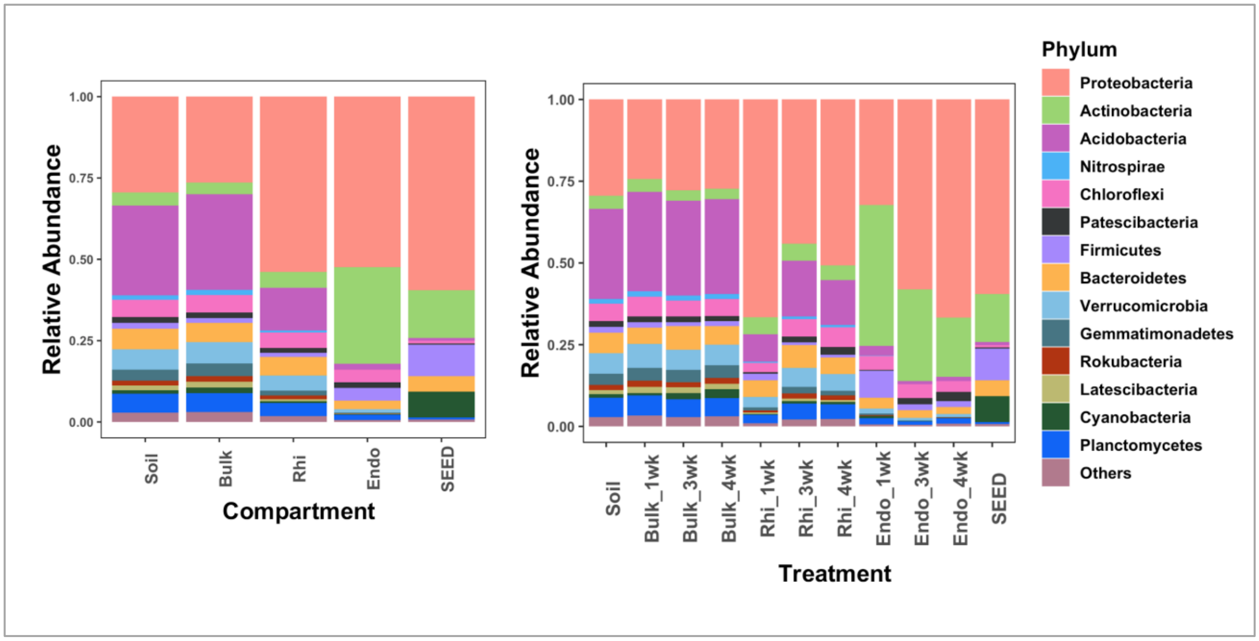


Fig. 4.4 Stacked barplot of the relative abundance of bacterial phyla.

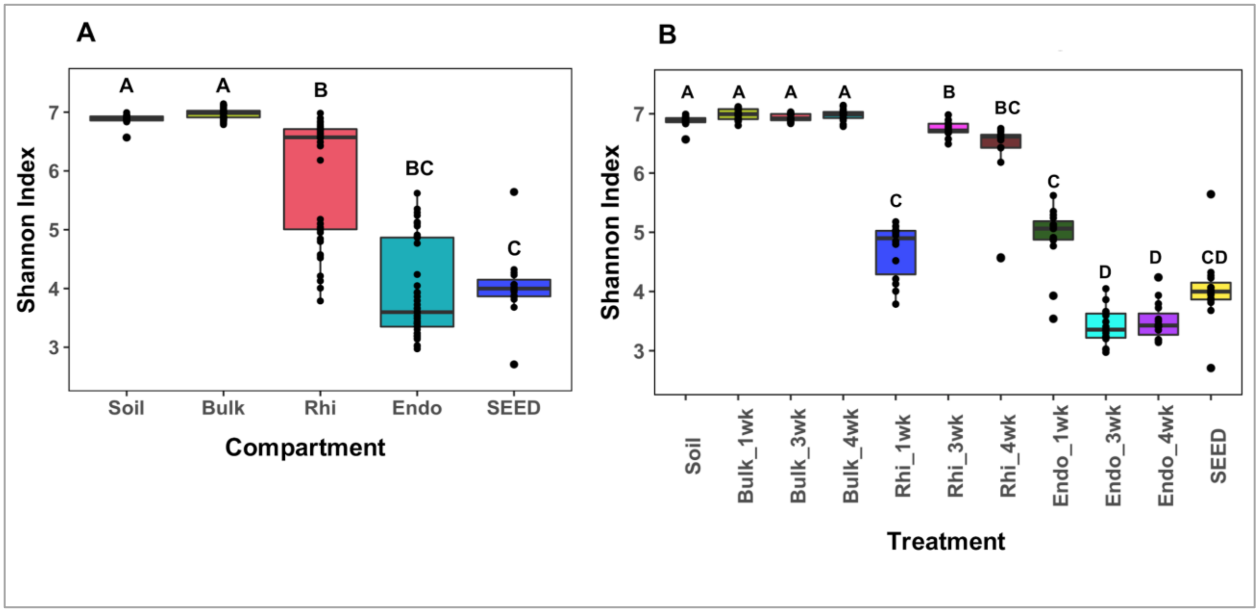


Fig. 4.5 Bacterial community diversity between compartments along with soybean development.

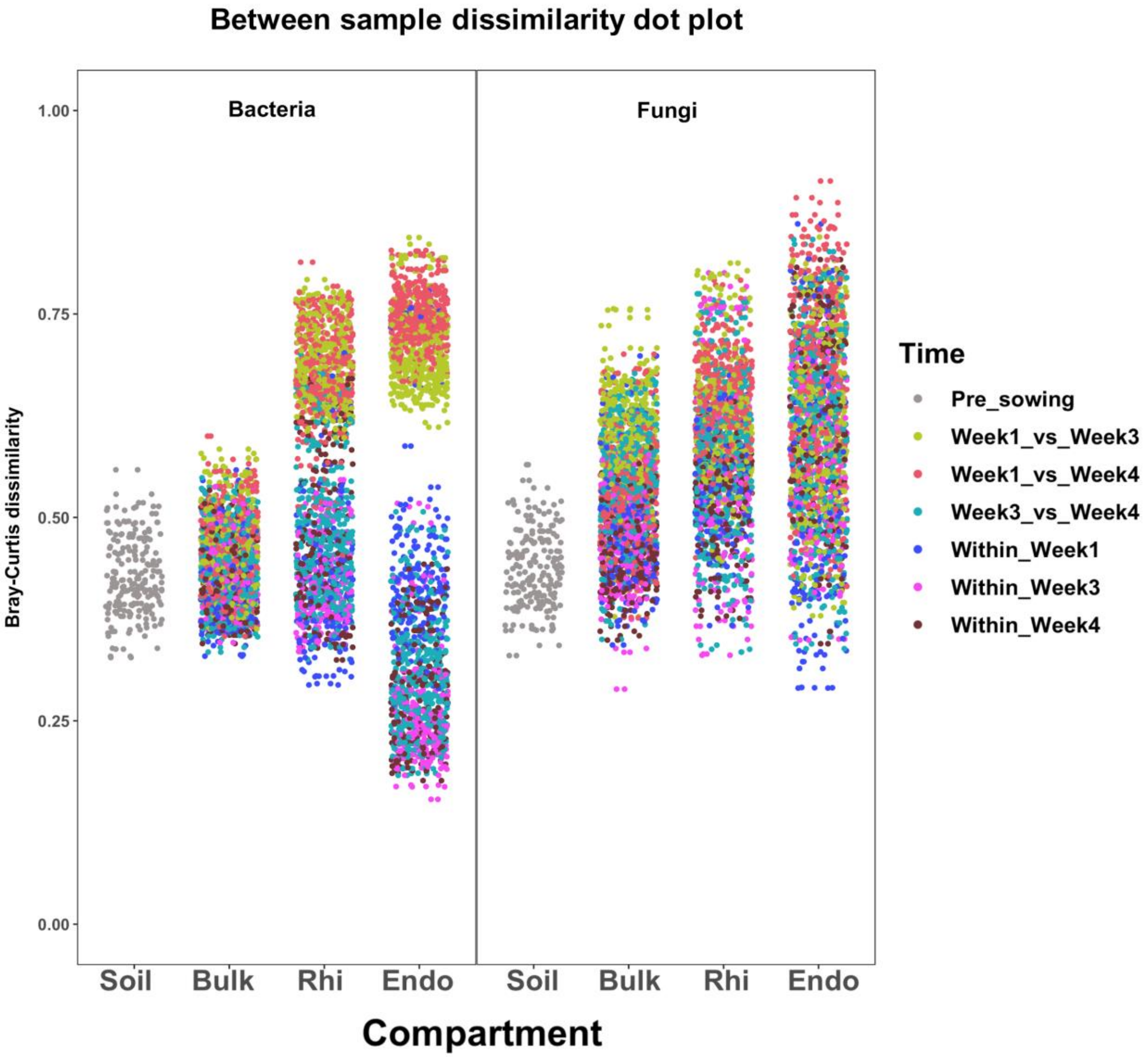


Fig. 4.6 Bray-Curtis dissimilarity between soybean development stages for each compartment.

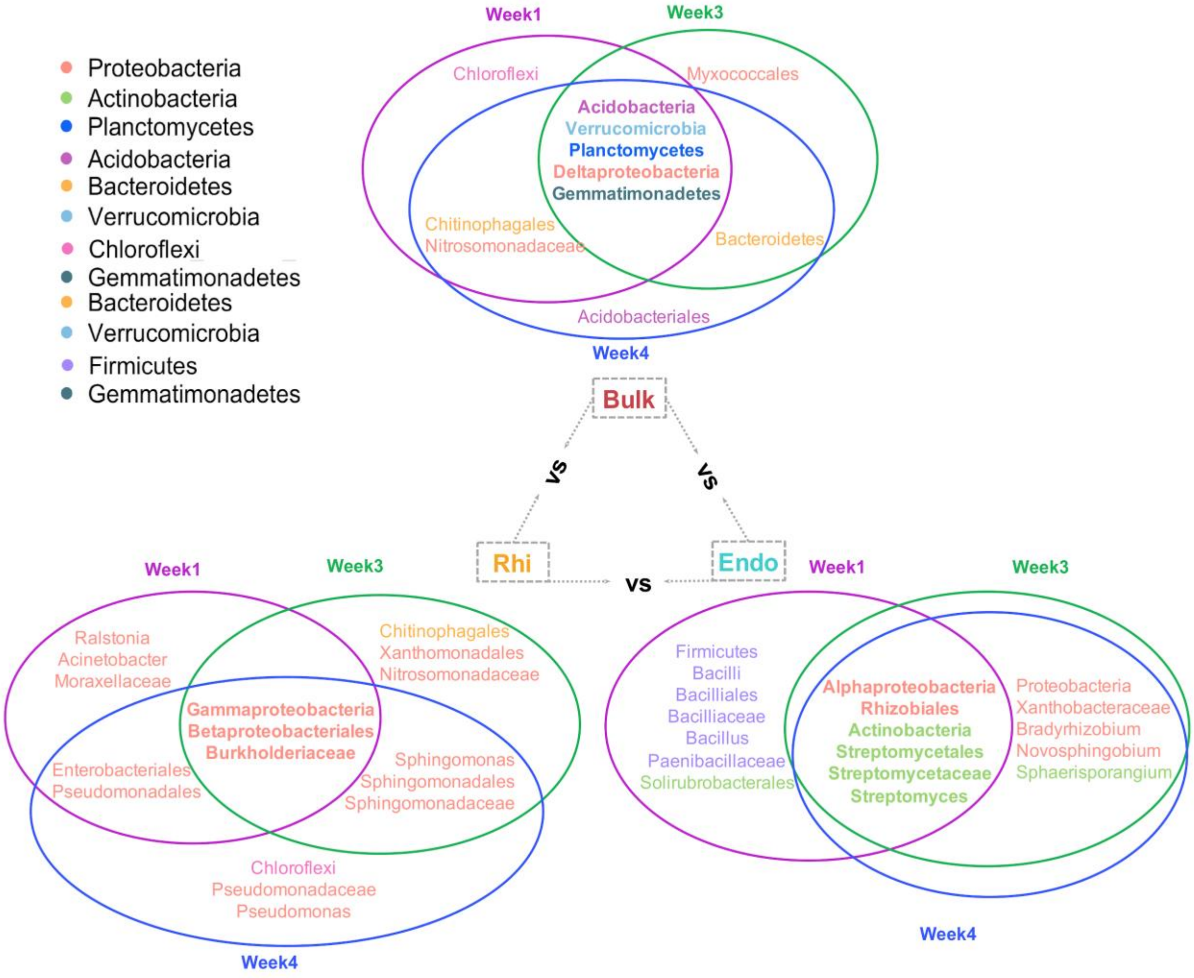


Fig. 4.7 Consistent and dynamic bacteria selection within each compartment.

All of the taxa listed were significantly enriched within specific compartment at specific development stages in comparison to the other two compartments. The color of the taxa was annotated based on their phylum level taxonomy.

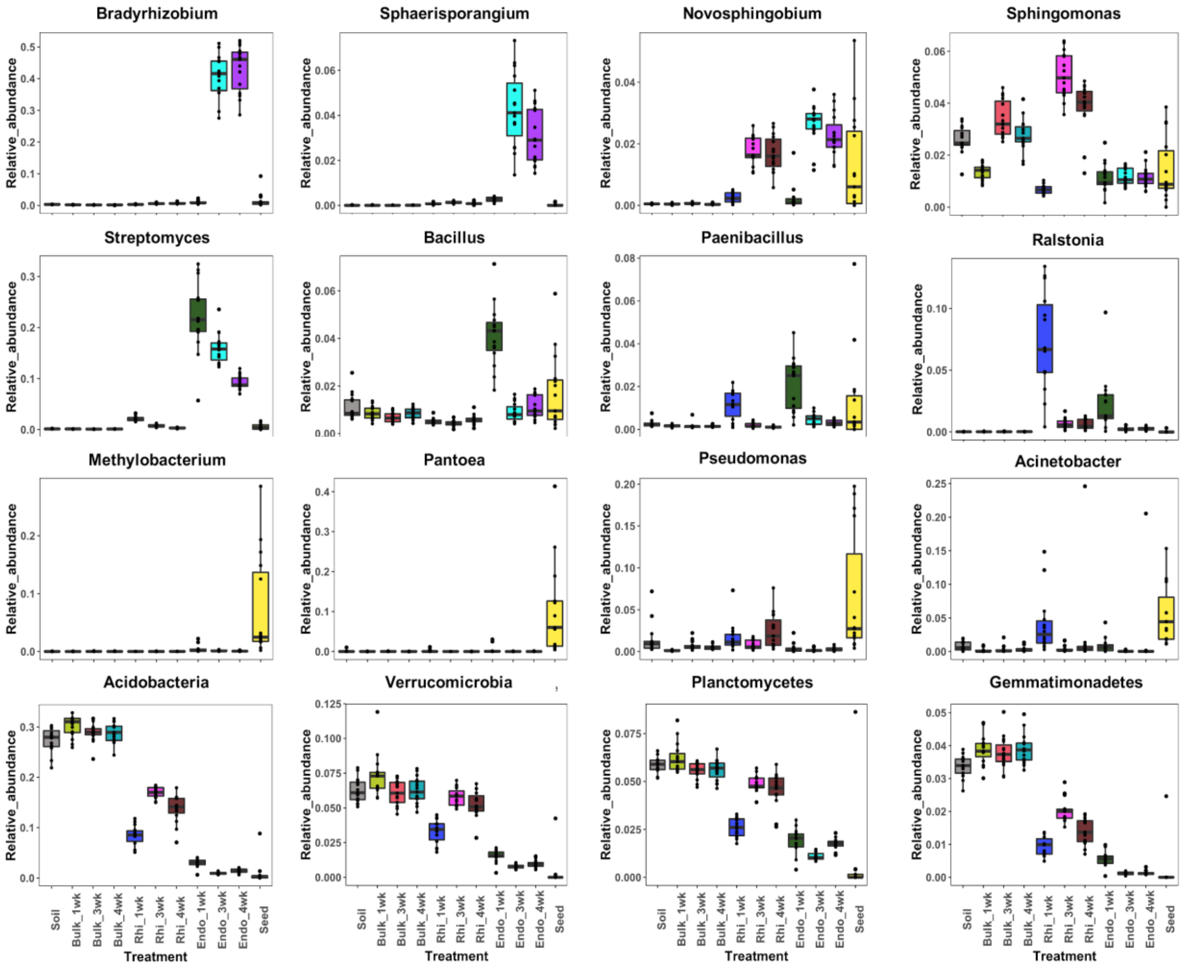


Fig. 4.8 The relative abundance barplot of bacterial taxa between treatments.

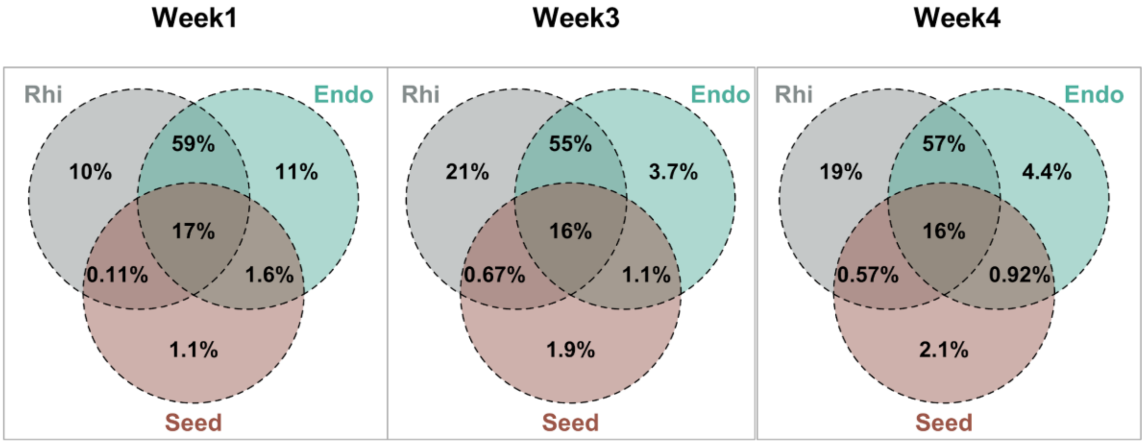


Fig. 4.9 Venn diagram for bacterial genera between rhizosphere, endosphere and seed compartments.

Fig. 4.10 Bacterial functional potentials between treatments.

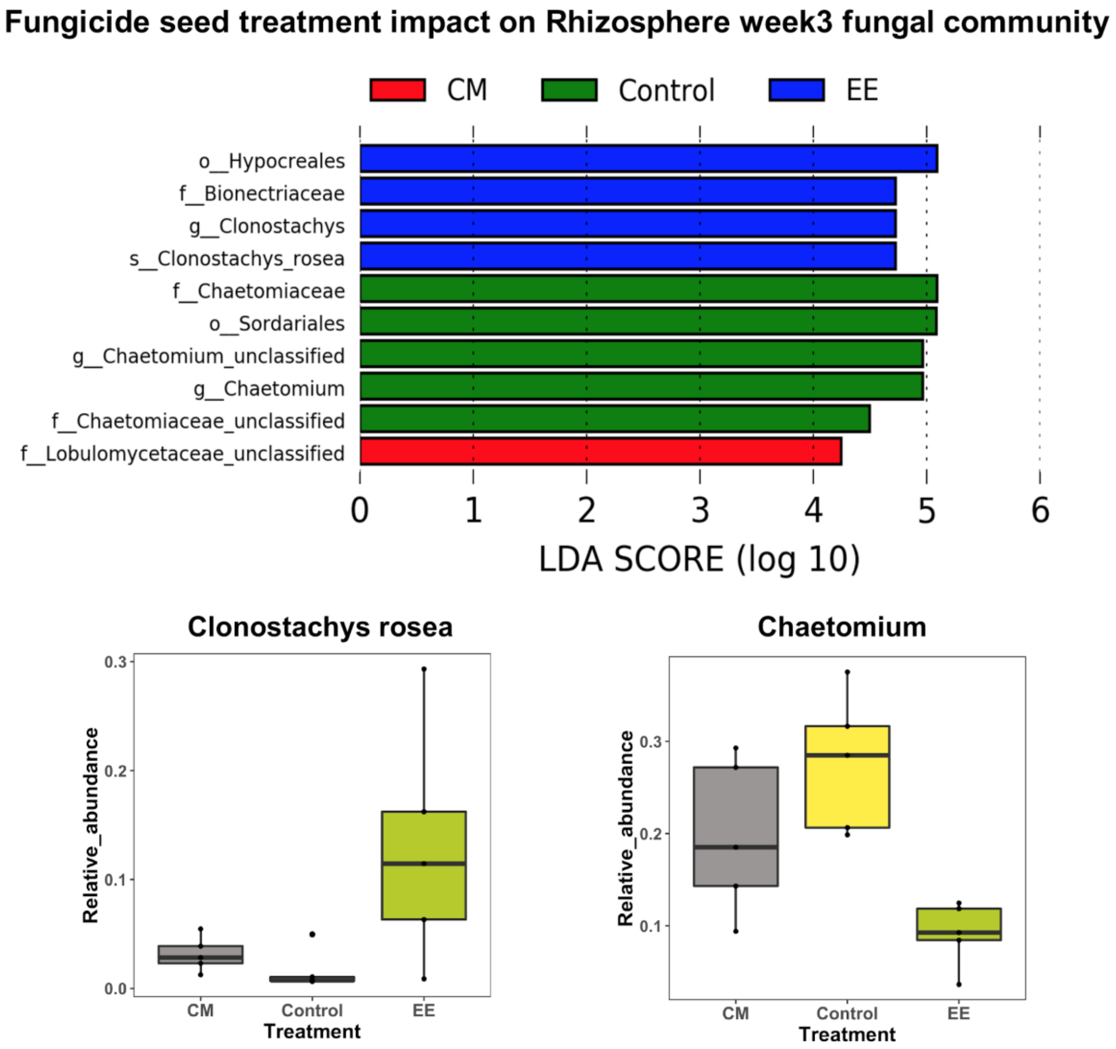


Fig. 4.11 Fungicide seed treatment impacts on fungal taxa for rhizosphere community at week3.

The differential abundance analysis between fungicide treatments were analyzed using LefSe software with LDA score = 4 as the threshold.

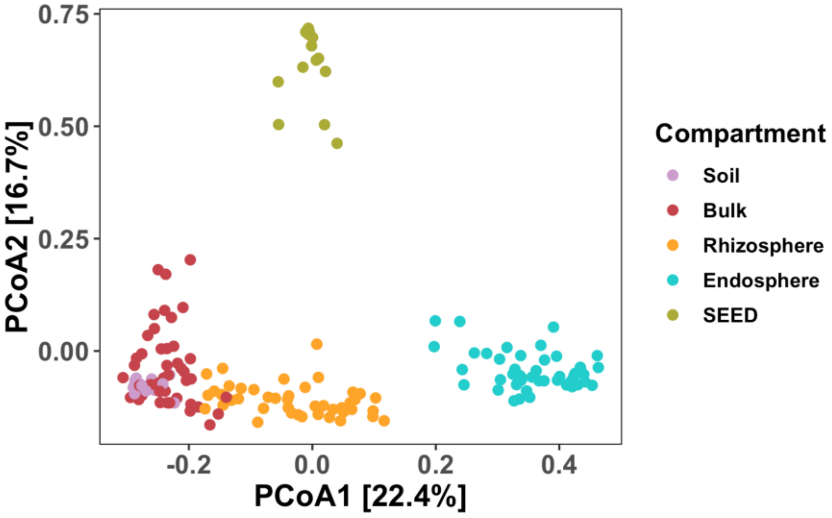


Fig. 4.12 Fungal community composition between compartments.

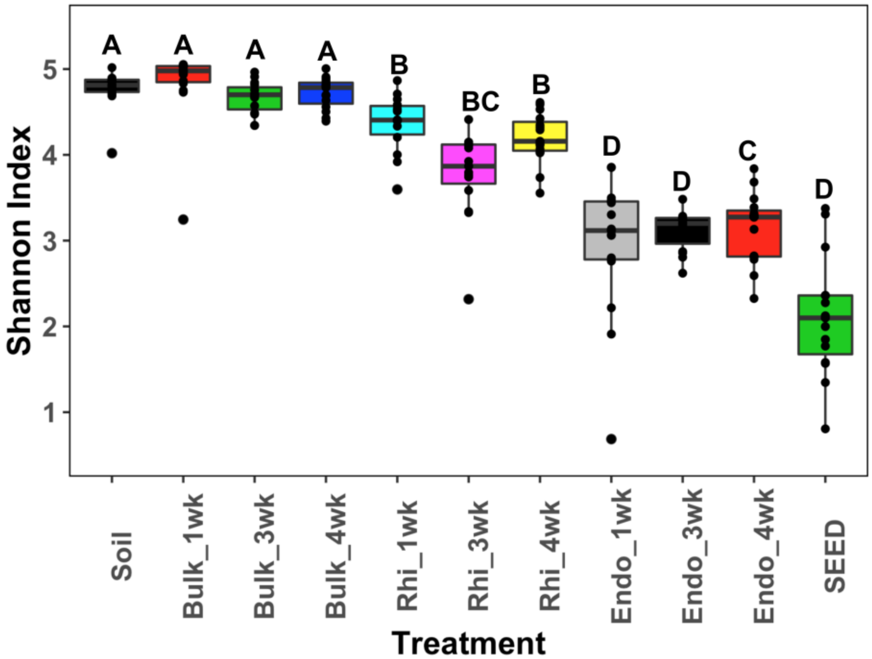


Fig. 4.13 Fungal community diversity between treatments.

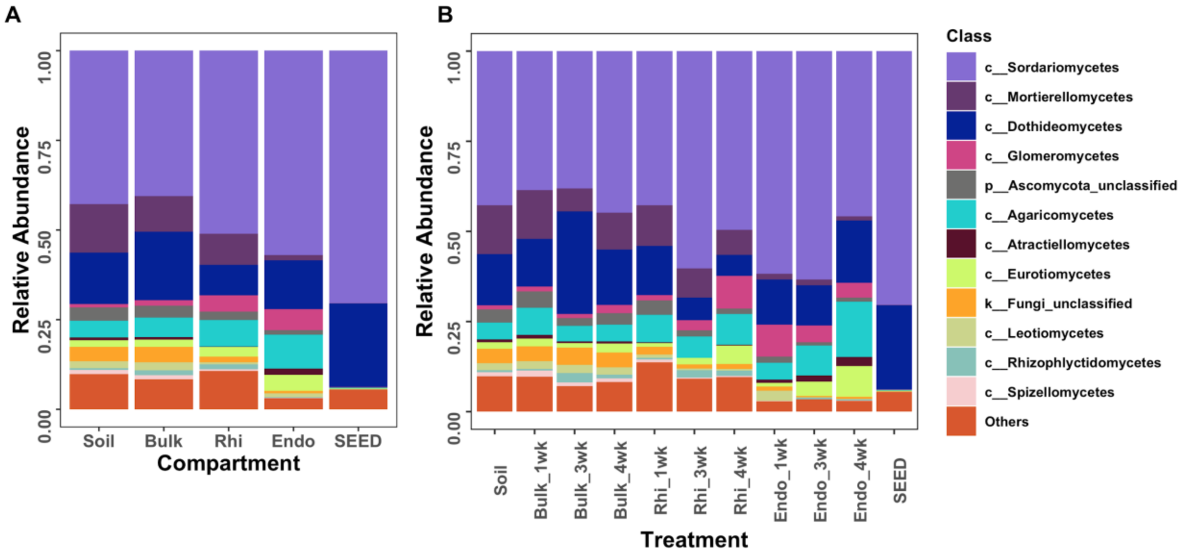


Fig. 4.14 Fungal phyla composition between compartments along with soybean developments.

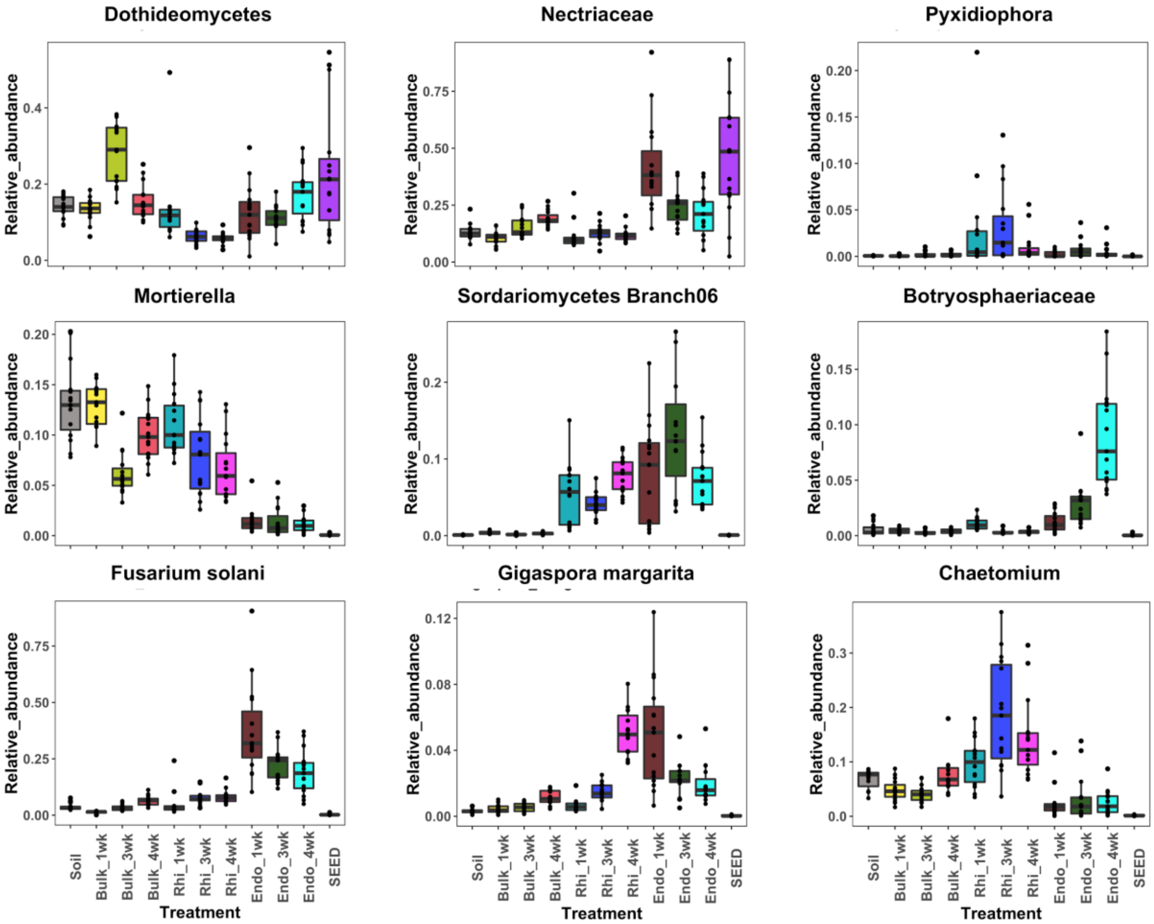


Fig. 4.15 The relative abundance barplot of fungal taxa between treatments.

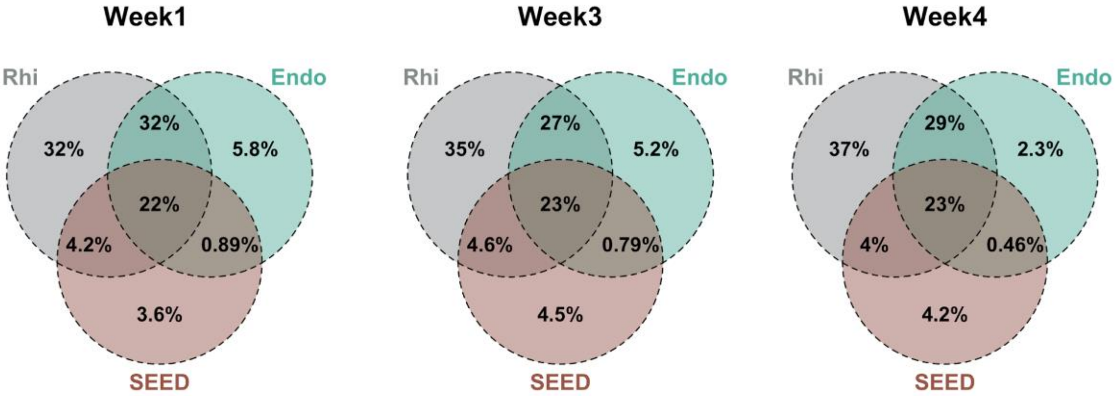


Fig. 4.16 Venn diagram for fungal genera between rhizosphere, endosphere and seed compartments.

Table 4.1 Fungicide seed treatment impact on bacterial and fungal community composition.

Group	Bacteria		Fungi	
	F-value	pvalue	F-value	pvalue
Soil, pre_sowing	1.02	0.38	1.04	0.36
Soil, bulk, 1wk	0.67	0.91	0.92	0.7
Soil, bulk, 3wk	0.95	0.53	1.03	0.37
Soil, bulk, 4wk	0.95	0.54	1.06	0.33
Rhizosphere, 1wk	0.79	0.79	1.18	0.16
Rhizosphere, 3wk	1.11	0.27	2.33	0.002**
Rhizosphere, 4wk	1.12	0.22	1.38	0.058
Endosphere,1wk	0.8	0.8	1.1	0.34
Endosphere, 3wk	0.84	0.64	1.4	0.083
Endosphere, 4wk	0.9	0.52	1.16	0.2
Seed	5.37	0.001***	1.52	0.079

Table 4.2 Compartment impacts on bacterial community - PERMANOVA marginal effects.

	Week1				**Week3**				**Week4**			
Factors	*df*	R2 (%)	*F*	P(>F)	*df*	R2 (%)	F	P(>F)	*df*	R2 (%)	F	P(>F)
Compartment	2	**58.50**	36.1	0.001***	2	**53.20**	47.4	0.001***	2	**64.80**	55.1	0.001***
Plots	14	**18.00**	1.59	0.02*	14	**15.10**	1.92	0.01**	14	**17.10**	2.08	0.003**
Read_depth	1	0.63	0.78	0.58	1	0.55	0.98	0.34	1	0.76	1.29	0.23
Residual	43	21.00	NA	NA	44	15.20	NA	NA	44	15.90	NA	NA

Table 4.3 Bacterial community changes along with soybean development-PERMANOVA marginal effects.

	Bulk				**Rhizosphere**				**Endosphere**			
Factors	*Df*	*R2 (%)*	*F*	*P(>F)*	*Df*	*R2 (%)*	*F*	*P(>F)*	*Df*	*R2 (%)*	*F*	*P(>F)*
Time	2	**10.95**	3.83	0.001***	2	**37.30**	17.10	0.001***	2	**58.70**	36.90	0.001***
Plots	14	**46.76**	2.34	0.001***	14	**27.50**	1.80	0.004**	14	***17.60***	1.58	0.057
Read_depth	1	1.29	0.90	0.563	1	0.81	0.74	0.592	1	1.96	2.46	0.066
Residual	27	38.55	NA	NA	26	28.40	NA	NA	27	21.50	NA	NA

Table 4.4 Compartment impacts on fungal community - PERMANOVA marginal effects.

	Week1				**Week3**				**Week4**			
Factors	*Df*	*R2 (%)*	*F*	*P(>F)*	*Df*	*R2 (%)*	*F*	*P(>F)*	*Df*	*R2 (%)*	*F*	*P(>F)*
Compartments	2	**21.40**	10.80	0.001***	2	**33.90**	17.4	0.001***	2	**27.00**	13.60	0.001***
Plots	14	**32.30**	2.33	0.001***	14	**29.30**	2.16	0.001***	14	**32.60**	2.36	0.001***
Read_depth	1	1.40	1.40	0.16	1	1.20	1.24	0.23	1	1.07	1.09	0.34
Residual	26	25.80	NA	NA	27	26.20	NA	NA	27	26.70	NA	NA

Table 4.5 Fungal community changes along with soybean development-PERMANOVA marginal effects.

	Bulk				**Rhizosphere**				**Endosphere**			
Factors	*Df*	*R2 (%)*	*F*	*P(>F)*	*Df*	*R2 (%)*	*F*	*P(>F)*	*Df*	*R2 (%)*	*F*	*P(>F)*
Time	2	**19.40**	7.06	0.001***	2	**16.17**	5.57	0.001***	2	**13.80**	4.32	0.001***
Plots	14	**38.50**	2.00	0.001****	14	**40.68**	2.00	0.001***	14	**38.30**	1.70	0.001***
Read_depth	1	1.60	1.16	0.230	1	1.57	1.08	0.360	1	2.42	1.51	0.065
Residual	27	37.20	NA	NA	26	37.74	NA	NA	27	43.30	NA	NA

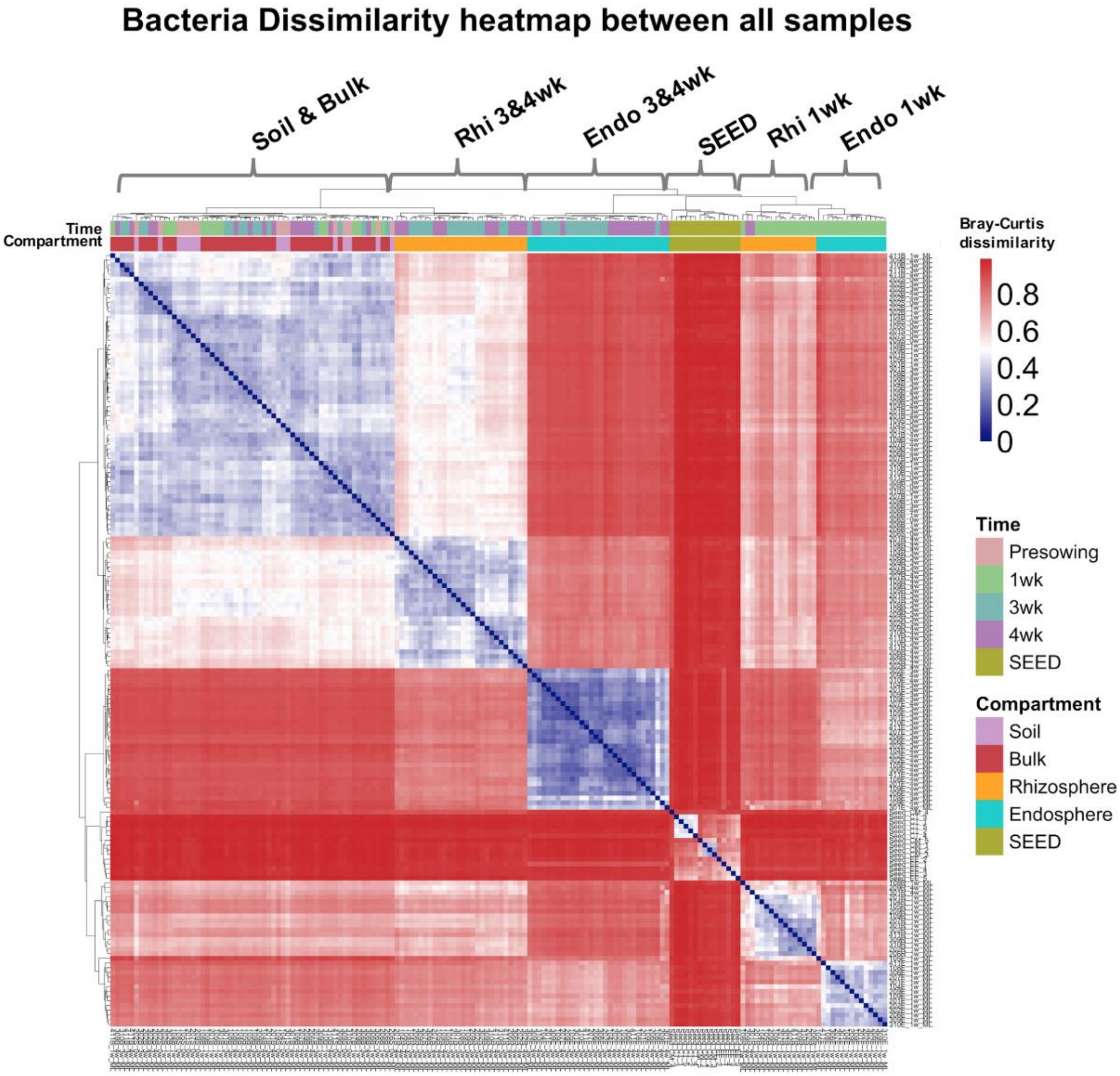


Fig. S4.1 Bacterial community dissimilarity heatmap across all samples.

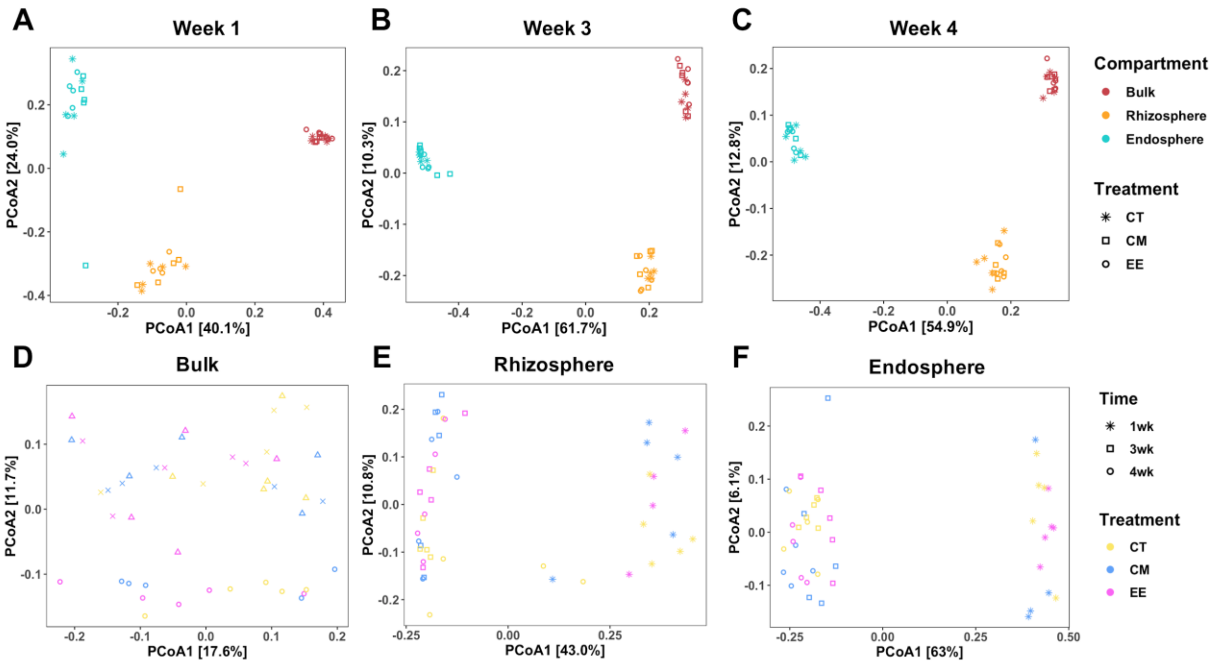


Fig. S4.2 Bacterial community composition between compartments and between development stages.

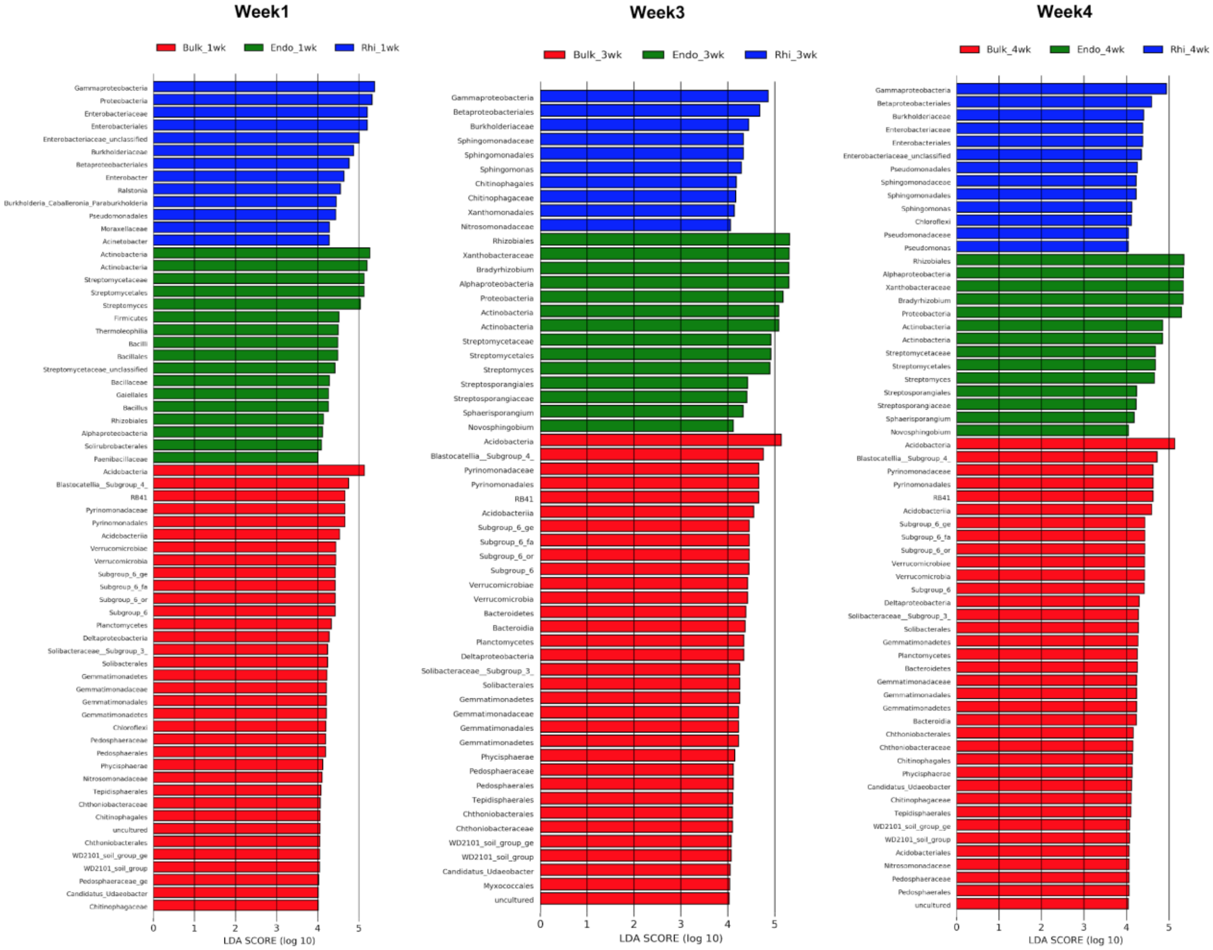


Fig. S4.3 Bacteria differential abundance analysis between compartments based on LefSe analysis at LDA score > 4.

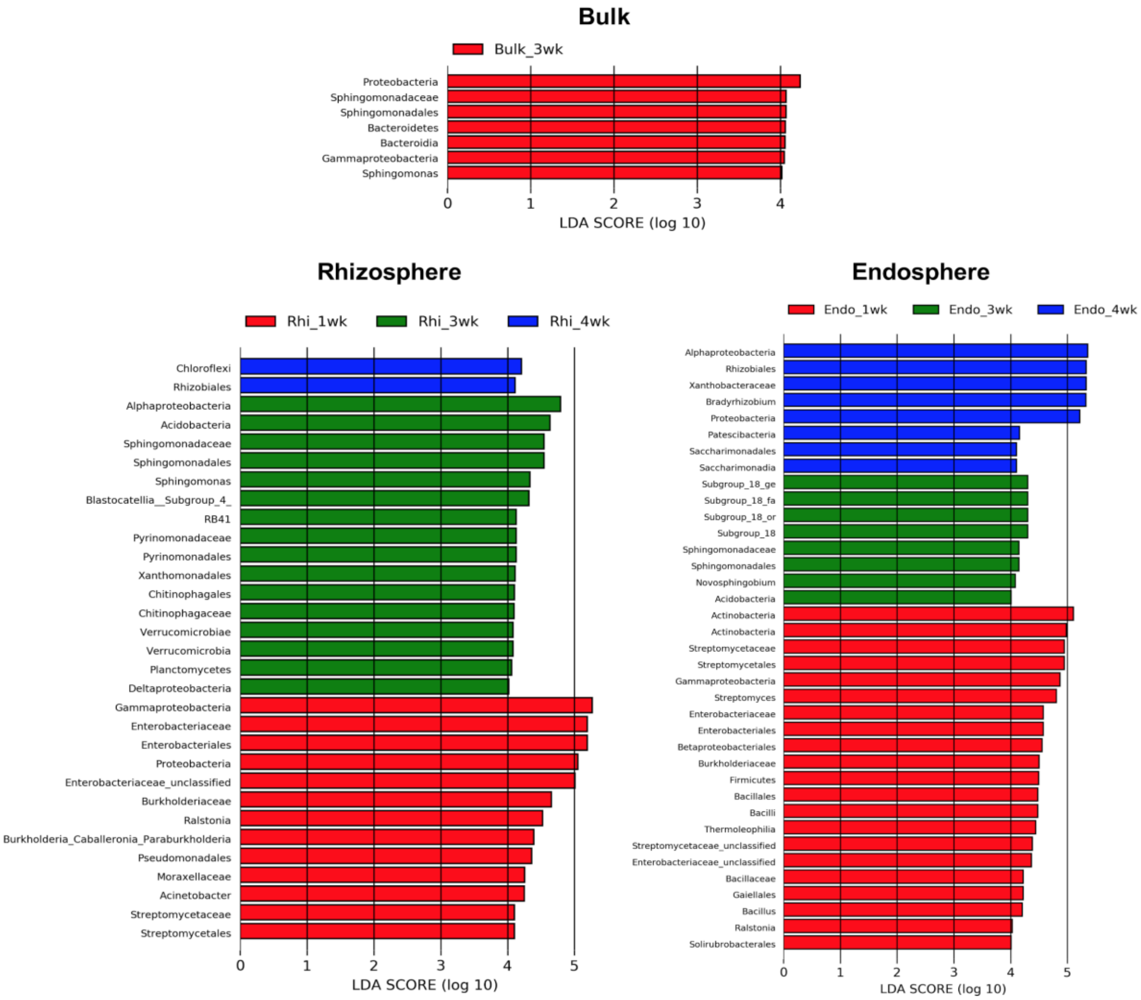


Fig. S4.4 Bacteria differential abundance analysis between different development stages at LDA score > 4.

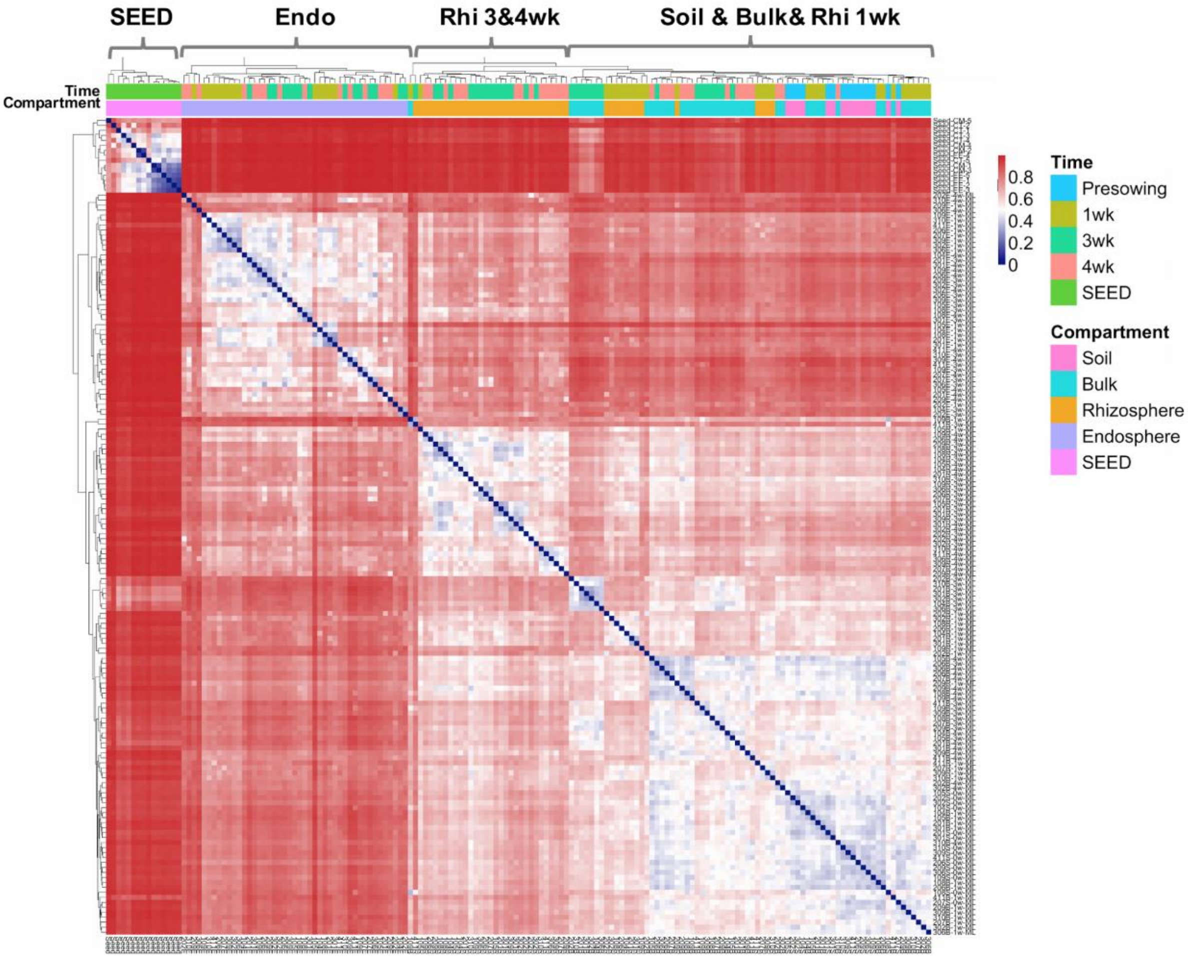


Fig. S4.5 Fungal community dissimilarity between samples.

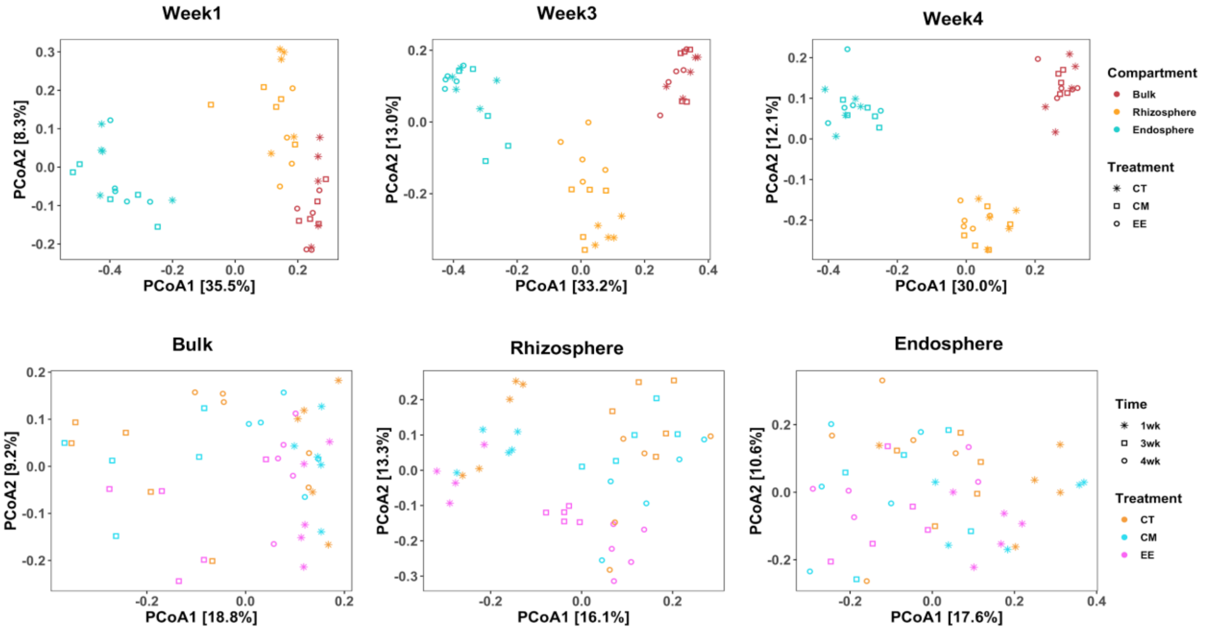


Fig. S4.6 Fungal community composition between compartments and between development stages.

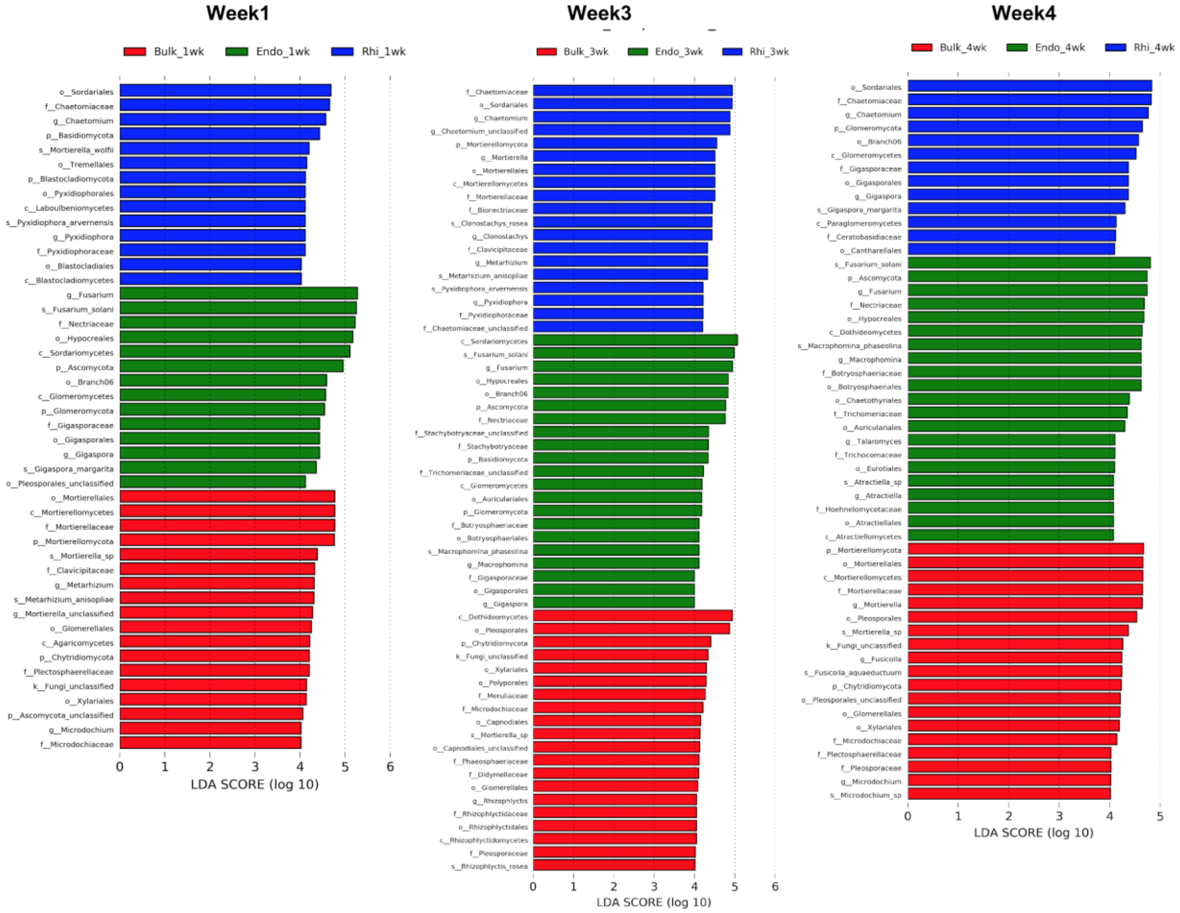


Fig. S4.7 Fungi differential abundance analysis between compartments based on LefSe analysis at LDA score > 4.

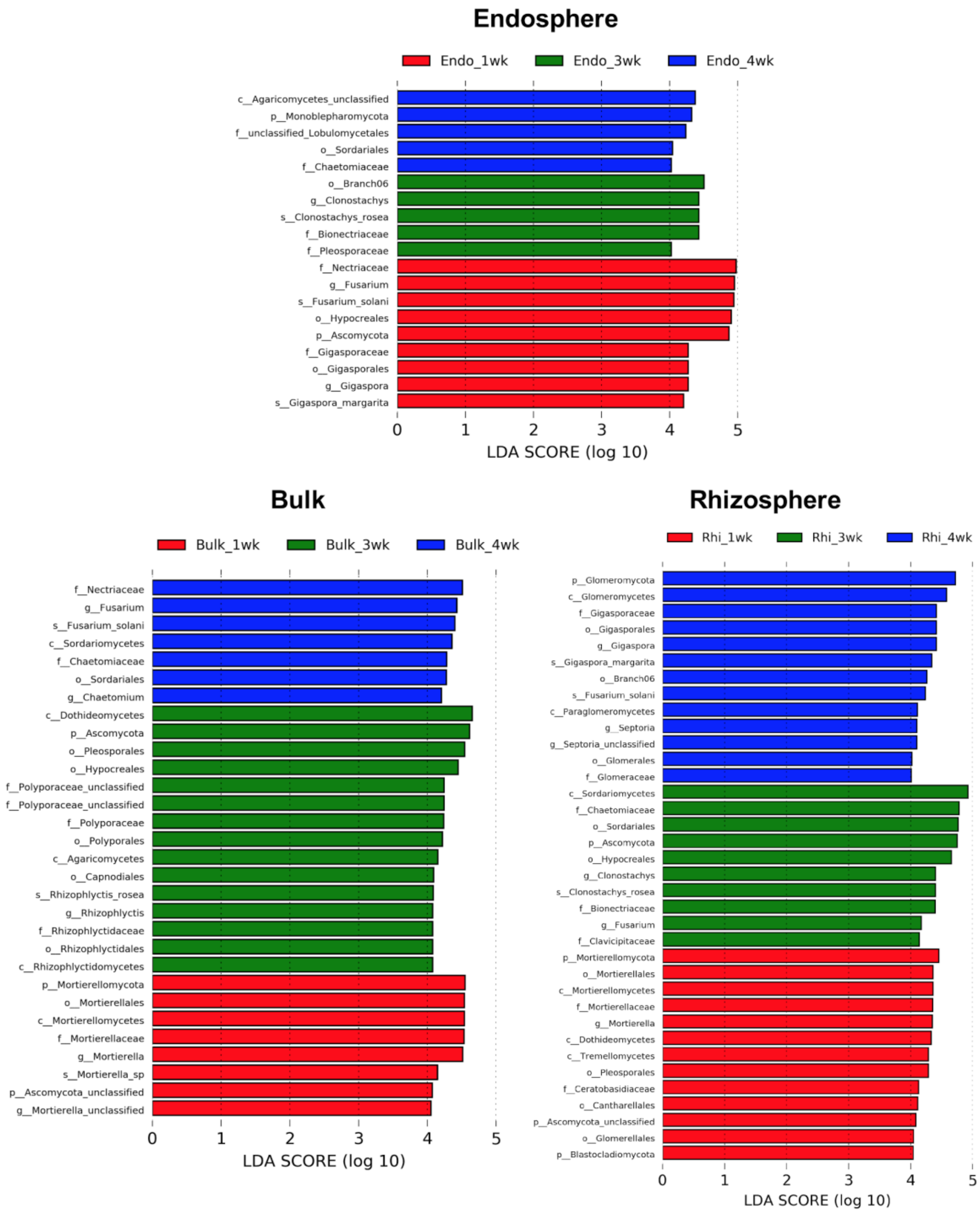


Fig. S4.8 Fungi differential abundance analysis between development stages based on LefSe analysis at LDA score > 4.

Table S4.1 Fungicide ingredients and composition.

	Ingredients	contents
Cruiser Maxx	Thiamethoxam	22.61%
	Mefenoxam	1.70%
	Fludioxonil	1.12%
	Other ingredients	74.57%
EverGol Energy	Prothioconazole	7.18%
	Penflufen	3.59%
	Metalaxyl	5.74%
	Other ingredients	83.49%

Table S4.2 Dominant bacteria genus associated with soybean seeds.

Phylum	Class	Order	Family	Genus	Relative abundance (%)
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea	10.07
Cyanobacteria	Oxyphotobacteria	unclassified	unclassified	unclassified	7.82
Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Methylobacterium	7.40
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	6.55
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	5.43

Chapter 5 Conclusion

Soybean is one of the most important crops worldwide, with a long history of breeding since domestication. The symbiotic interaction between soybean and nitrogen-fixing rhizobia has been extensively investigated as a key process that provides nutrition to the plant and improves soil fertility. However, soybean performance as a crop depends on a much more extensive plant-soil-microbe system. In this study, both microcosm- and field-based characterization of the soybean-associated microbial community was conducted from the integrative perspective of the host plant, soil, and seed, providing an unprecedented picture of soybean root microbiome assembly and underlying driving factors. As a community sequence-based characterization of a complex system, this research provides a broad overview of what microbes associate with soybeans and illuminates some key factors that drive this association. This data can be mined to develop specific fundamental hypotheses about the role of individual microbial taxa in soybean physiology and performance as a crop. This foundational knowledge is building toward breeding- and biocontrol-assisted sustainable agriculture via maximizing the potential beneficial soybean-microbe interactions.

Our findings suggest that soybean develops a reproducible set of microbial taxa in the rhizosphere, derived from the microbes available in the surrounding soil. Based on our results and previous studies, this subsetting from the soil microbial pool to a specialized rhizosphere community results from specialized root exudates and secondary metabolites. This plant process initiates, maintains, and modifies the symbiotic interaction with nitrogen-fixing rhizobia and arbuscular mycorrhizal fungi as well as many other taxa. Specifically, *Proteobacteria* and *Actinobacteria* were consistently enriched in the soybean rhizosphere, while, *Acidobacteria Verrucomicrobia* and *Planctomycetes* were significantly depleted within the rhizosphere in comparison to bulk soil. By testing different soil types and soybean genotypes, we found the bacterial community composition in the soybean rhizosphere is predominantly determined by the soil indigenous microbial pool. The influence of soil microbial pool was reflected by the selective enrichment of *Firmicutes* and *Bacteroidetes* in the soybean rhizosphere growing in agriculture and forest soil, respectively. We found soybean genotype also influenced rhizosphere bacterial composition, although to a lesser extent than soil background. We found comparatively larger differences between the progenitor species (*Glycine soja)* and other modern cultivars than among the modern cultivars, suggesting domestication may have altered or reduced variability in rhizosphere microbiome assembly.

To understand how soybean influences the rhizosphere microbiome through root exudates, we examined the role of a plant hormone family known to be exported from roots to the rhizosphere, strigolactones. Strigolactones are involved in plant physiology, development, stress tolerance and, plant-microbe communications. While previous research has highlighted strigolactones' role in regulation and recruitment of mycorrhizal fungi and, to some degree, rhizobia, the relationship of strigolactones to other root-associated taxa has been largely unexamined. To fill this gap, we characterized soybean rhizosphere microbial community composition in response to the overexpression of strigolactone synthesis and signaling related genes. Our results indicate small but significant impacts by altered expression of both strigolactone biosynthesis (Max1) and perception genes (D14 and Max2) on rhizosphere bacterial and fungal communities. The bacterial community was more responsive to the overexpression of strigolactone related genes in comparison to the fungal community. Despite the significant results in regard to community composition, we did not observe a significant change in their functional potentials.

The seed microbiome has been suggested as an important factor for plant microbiome assembly and plant health by providing the initial inoculum for the root microbiome during the early germination stage. However, we still lack information in terms of how robustly these seed-carried microbes are kept within plant roots along with soybean development in comparison to the colonization by the soil-derived microbe. In addition, there is very limited information about how fungicide seed treatment, a common agricultural practice, impacts the root microbiome assembly process. To answer this question, we comprehensively characterized field soybean root microbiomes from bulk, rhizosphere, endosphere, and seed compartments along with the early development of seedlings (1 week to 4 weeks) with and without fungicide treatment. Our results demonstrated that soybean selectively recruited specific bacterial and fungal taxa into the rhizosphere and endosphere from surrounding soil in a development-dependent way. At one week after sowing, soybean seedlings harbored a unique bacterial community in comparison to that of week three and week four, with *Gammaproteobacteria* and *Actinobacteria* being the most dominant taxa in rhizosphere and endosphere, respectively, at week1. Both were strikingly decreased at week three and four. Most seed dominant bacterial taxa, such as *Pantoea*, *Pseudomonas* and *Methylobacterium*, were dramatically decreased within soybean roots after one week of growth in soil. However, a few taxa originating from the seed remained after germination, such as *Bacillus* with a high abundance within the root but not in the rhizosphere. In contrast to the dynamic and stringent structuring of bacteria within each compartment, fungal community changes were less evident between compartments or between time points, while between plot variation was comparatively high. The bacterial functions of inositol degradation and seed mucilage (D_galacturonate) degradation pathway were more abundant in the soybean rhizosphere at week one, while aromatic compound degradation related pathways were enriched for endosphere bacterial community, which greatly diversified along with soybean development. Fungicide seed treatment impacts were insignificant for the bacterial microbiome, but caused, a minor decrease of the fungal genus *Chaetomium* in the soybean rhizosphere at week 3.

Overall, our studies provide a comprehensive understanding of soybean root microbiome assembly from the plant, soil and seed perspectives by taking advantage of the high depth of sequencing data together with high numbers of biological replicates. Meanwhile, we appreciated some of the limitations of our current studies, which motivated us to highlight a couple of potential research directions that worth further investigation. From the soil aspect, more detailed soil physico-chemical measurements, especially those that are indicative of soil nutrient status and soil structure, will help to infer the underlying soil factors that drive the divergent root microbiome assembly. Since root morphology and root exudates composition actively mediate associated microbial community, advanced *in situ* root imaging together with targeted/ untargeted metabolomics along with soybean development between different cultivars grown in different soil warranted for further study. Considering the multi-function feature of strigolactones, a more detailed investigation using stable transgenic lines, with and without functional destruction in AMF/nodule formation together with strigolactone quantification, will provide a clearer picture of the direct and indirect impact exerted by strigolactones. In addition, a more closed examination of root microbiome assembly dynamics along with seed germination and seedling establishment in both axenic, synthetic community-based reduced experimental system, and natural soil systems will help to understand the relative importance of seed-carried microbes versus soil-derived microbes during soybean root microbiome, which will help to guide microbe inoculant-based biocontrol application for sustainable agriculture.

Vita

Fang Liu comes from Shandong province in China. She graduated from Sihui High shool in 2008 and went to attened Liaocheng University. In 2012, she finished her study and graduated with a Bachelors degree in Biology. After graduation, Fang went to the Institute of Applied Ecology, Chinese Academy of Science to continue her master study. In 2015, she graduated with a Master of Science in Soil Ecology and went to University of Tennessee, Knoxville for Ph.D. study focused on soybean microbiome work. During her Ph.D. study, Fang had multiple opportunities to attend conference and presented her work. She also got one paper published in BMC microbiology and another two drafts waiting to be submitted soon.