MECHANISMS TO MANAGEMENT: HARNESSING PLANT-MICROBIAL INTERACTIONS AND SOIL HEALTH FOR SUSTAINABLE AGRICULTURE

By

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ABSTRACT

There is an urgent need to address challenges with environmental degradation and climate change in our agricultural landscapes, and the solution may lie with soil microbes. The soil microbes living in close association with plant roots, in the rhizosphere, play a central role in nutrient cycling, carbon sequestration, and plant growth and, therefore have significant promise for agriculture. Compared to annual crops, perennial cropping systems, such as those used for cellulosic bioenergy, do more for addressing carbon sequestration and soil health. However, to harness the benefits of microbes in these systems, we need to first understand the primary factors impacting their assembly. Many studies show microbial assembly is largely mediated by the plant host, but these studies often focus on isolated plants, and do not consider how neighboring plant interactions may also alter microbiome assembly. Furthermore, for soil biology to be an agricultural solution it is also essential that their benefits are clear and align with farmers' management goals. Studies show that farmers value soil biology and soil health more broadly, but how this guides their management decisions is unknown. To this end, in my dissertation, I use microbial ecology (Chapters 1-3) and social science (Chapter 4), to investigate how plant-microbial interactions and farmer perspectives can be harnessed for sustainable agriculture.

In my first three chapters I examine how switchgrass (*Panicum virgatum* L.), a candidate bioenergy crop, mediates the assembly of its root and rhizosphere microbiome, considering two factors: genotype and neighborhood context. In Chapter 1 I asked if, like plant species, plant genotypes also associate with distinct microbiomes. Using an established field experiment with twelve mature switchgrass cultivars, I found that genotypes have subtle, though significant effects on their rhizosphere microbiomes, and that root traits contribute to this variation. Next, in Chapters 2 and 3, I asked if and how a host plant's microbiome changes with different neighbor plants. To do this, I used two different greenhouse experiments where a focal switchgrass plant was neighbored by different species. In Chapter 2, I show that neighbor identity explains 21% of the variation in the focal plant's rhizosphere community. Changes in the focal plant's root exudates, as well as spillover of microbes from a larger, more competitive neighbor, contributed to the microbiome shifts. In Chapter 3, I disentangle the relative role of microbial spillover versus the host plant in mediating the previously observed neighborhood effects by using specialized plant growth systems called rhizoboxes with root barriers. Here, neighbor identity altered the root microbiomes, but not rhizosphere communities, which also did not differ among the plant species. These results suggest that the host plant does play a role in mediating neighborhood effects in the roots, but shifts in the rhizosphere depend upon each neighbor

species harboring a distinct microbiome in the first place. My first three chapters show that there is not one switchgrass microbiome, and that microbial assembly is influenced by plant genotype and neighborhood context. Both factors should be considered as we seek to understand plant-microbial studies in natural settings.

Finally, in Chapter Four, I ask how farmers perceive, evaluate, and understand soil health. Using surveys and interviews I found that Michigan farmers have a complex understanding of soil health, and that soil biology is a top consideration, but that it is challenging for farmers to link this knowledge to management decisions. The interviews also revealed several salient research and outreach opportunities that could help farmers more intentionally fit soil health into their management decisions, such as identifying faster-responding indicators of soil biological health or discussing soil health in terms that resonate with farmers' mental models. Altogether, my dissertation shows how mechanistic studies and farmer perspectives each provide novel insights for the potential role of soil biology in sustainable agriculture.

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INTRODUCTION

There is an urgent need to transform our agricultural landscape to reduce environmental harm and mitigate climate change, and recent studies suggest that the solution lies with the soil (Lal, 2004; Chaparro et al., 2012). The soil provides critical ecosystem services, such as nutrient cycling, carbon sequestration, and water infiltration (Wall et al., 2012). However, these services vary across cropping systems and recent studies suggest that, compared to annual row crop systems, perennial crops planted for cellulosic bioenergy have greater potential for sequestering greenhouse gases and building soil carbon (Gelfand et al., 2013; Robertson et al., 2017; McGowan et al., 2019). Studies suggest that the positive effect of perennials on ecosystem services is in part due to their deep rooting systems (Lemus and Lal, 2005), as well as associations with beneficial microbiomes (Hestrin et al., 2021). The soil microbes living in close association with plant roots, in the rhizosphere, play a particularly important role in nutrient cycling, carbon sequestration, and even plant growth (Philippot et al., 2009; Berendsen et al., 2012; Chaparro et al., 2012). However, the ability to harness the potential of these microbes in agricultural landscapes, demands an understanding of their assembly, as well as farmer perceptions of their dynamics and role in agriculture.

Plant hosts play a primary role in the assembly of rhizosphere communities, in part through secreting species-specific signals in the form of root exudates (Berg and Smalla, 2009), but many other factors also impact the rhizosphere and it can be challenging to determine the relative role of the host plant amidst this complexity. For instance, the initial pool of microbes in the bulk soil (Vieira et al., 2019), changes in abiotic conditions (Fierer et al., 2007; Naylor, 2017), and even signals exchanged between interacting plants (Chen et al., 2020) can alter microbial community structure. Because of this complexity, many studies on plant-microbial interactions use simplified systems, whereby a single plant species is grown in controlled conditions. Even when studies do move to the field, they often ignore important factors that may influence microbial assembly, such as differences in neighborhood context. Plant neighbors have been shown to alter a host plant's microbial community (Mummey et al., 2005; Hausmann and Hawkes, 2009; Hortal et al., 2017a; Mony et al., 2021), but the effect can be weak compared to other factors (Horn et al., 2017; Vieira et al., 2019), and the role of the host plant in mediating these changes is unknown. In agricultural systems, plants do not grow in isolation and, therefore, investigating the processes that mediate microbial assembly in more complex environments, like with different plant neighbors, is a critical step to being able to harness the benefits of soil microbes on-farm.

Even though scientists may identify beneficial plant-microbial interactions that could increase plant growth or resiliency, farmers have the ultimate power for deciding how soils are managed. Farmers weigh many factors, such as economic risk and social pressure, when deciding how to manage their land (Carlisle, 2016; Prokopy et al., 2019), but little is known about how their perspectives of the soil itself informs their management decisions. Previous interviews with farmers show that many identify as stewards of the soil (Roesch-McNally et al., 2018), and that they value soil biology (Romig et al., 1995; Irvine et al., 2023), but that they are also skeptical of new microbial products (Doll et al., 2020). Therefore, deeper studies of farmers' perspectives on, as well as management of, soil health and soil biology could help inform how scientific studies on plant-microbial interactions are already implemented, or could be integrated, on-farm.

To fill these gaps, I address two broad questions in my dissertation. First, to what degree do plant hosts mediate rhizosphere assembly in complex environments, including in those with different plant neighbors? And second, how do farmers perceive and manage for soil health? I use an interdisciplinary approach to address these questions, examining how plant-microbial interactions (Chapters 1-3) and farmers perspectives (Chapter 4) can inform the potential role of soil biology in sustainable agriculture.

In my first three chapters, I investigate the degree to which a host plant, switchgrass (*Panicum virgatum* L.), mediates the assembly of its root and rhizosphere microbiomes. Switchgrass is a native tallgrass prairie species, as well as a candidate cellulosic bioenergy crop (McLaughlin and Kszos, 2005). Studies suggest that microbial communities contribute to its feasibility as a bioenergy crop (Hestrin et al., 2021), including its ability to grow in less-fertile, drought-prone soils that do not compete for food production (Gelfand et al., 2013). For instance, free-living nitrogen fixers may increase switchgrass's ability to access nutrients (Roley et al., 2018, 2019) and fungal endophytes can increase its resistance to drought stress (Ghimire et al., 2009; Ghimire and Craven, 2011). It is also possible that these microbes contribute to the wide genotypic variability observed in switchgrass yields and ability to tolerate stress (Casler et al., 2017; Stahlheber et al., 2020). If so, then understanding their assembly could also help inform genotype-specific breeding programs. Furthermore, microbial interactions are also suggested to contribute to variation in soil carbon accrual in switchgrass cropping systems (Tiemann and Grandy, 2015; Kravchenko et al., 2019). For instance, carbon accumulation is often greater in diverse perennial polycultures than in monocultures (Sprunger and Robertson, 2018; Yang et al., 2019), perhaps because interplant carbon transfer, a process which can be mediated by microbial communities, differs among different neighbor plants (Kravchenko et al., 2021). Switchgrass is cultivated in both monocultures and diverse polycultures, but the degree to which plant neighbors influence switchgrass microbiome assembly is unknown and needs elucidated.

Building on previous literature and gaps in our understanding of switchgrass microbiomes, I examine how switchgrass mediates the assembly of its root and rhizosphere microbiome, considering two factors: genotype and neighborhood context. In Chapter 1, I investigate the relative role of plant genotype, as well as root traits, in structuring switchgrass rhizosphere and endosphere communities using an established field experiment with twelve mature switchgrass cultivars (seven years established). In Chapters 2 and 3, I examine how neighborhood context influences a host plant's microbiome assembly using two greenhouse experiments. In both studies, I explore how different neighbor species impact a focal switchgrass plant's microbiome and, if so, to what extent the host-plant mediates these change through root exudates. In the second greenhouse study (Chapter 3), I used rhizoboxes with root barriers and fine-scale sampling to more clearly differentiate the relative role of the host- and neighbor-plants in mediating neighborhood effects. Together, the first three chapters inform the degree to which switchgrass mediates the assembly of its microbiome and, furthermore if plant-microbial studies on isolated plants can be used to predict assembly processes in more complex growing contexts.

Finally, in Chapter 4, I work with Michigan row crop farmers to understand how their perceptions of soil biology and, soil health more broadly, inform their management decisions. To do this, I use surveys, interviews, and mental models to investigate how Michigan farmers perceive, evaluate, and manage soil health. Though this work focuses on Michigan farmers, it provides broader recommendations for research and outreach that could help farmers from many regions more intentionally use soil health to guide management. In summary, my dissertation uses a novel, interdisciplinary approach and reveals how mechanistic studies and farmer perceptions both play a role in realizing the potential for soil biology to create a more sustainable agricultural landscape.

CHAPTER ONE: INTRASPECIFIC VARIABILITY IN ROOT TRATIS AND EDAPHIC CONDITIONS INFLUENCE SOIL MICROBIOMES ACROSS 12 SWITCHGRASS CULTIVARS[1]

ABSTRACT

Microbial communities help plants access nutrients and tolerate stress. Some microbiomes are specific to plant genotypes and, therefore, may contribute to intraspecific differences in plant growth and be a promising target for plant breeding. Switchgrass (Panicum virgatum L.) is a potential bioenergy crop with broad variation in yields and environmental responses; recent studies suggest that associations with distinct microbiomes may contribute to variation in cultivar yields. We used a common garden experiment to investigate variation in 12 mature switchgrass cultivar soil microbiomes and, further, to examine how root traits and soil conditions influence microbiome structure. We found that average root diameter varied up to 33% among cultivars and that they associated with distinct soil microbiomes. Cultivar had a larger effect on the soil bacterial than fungal community, but both were strongly influenced by soil properties. Root traits had a weaker effect on microbiome structure, but root length contributed to variation in the fungal community. Unlike the soil communities, the root bacterial communities did not group by cultivar, based on a subset of samples. Microbial biomass carbon and nitrogen and the abundance of several dominant bacterial phyla varied between ecotypes, but overall the differences in soil microbiomes were greater among cultivars than between ecotypes. Our findings show that there is not one soil microbiome that applies to all switchgrass cultivars, or even to each ecotype. These subtle but significant differences in root traits, microbial biomass, and the abundance of certain soil bacteria could explain differences in cultivar yields and environmental responses.

INTRODUCTION

Plants associate with microbial communities that help them access resources and tolerate stress (Pérez-Jaramillo et al., 2016; Jiang et al., 2017). Some microbial communities are associated with specific plant genotypes (Emmett et al., 2017; Jiang et al., 2017; Pérez-Jaramillo et al., 2017; Adam et al., 2018) and so have the potential to be targets of plant breeding programs and inform crop choices (Mueller and Sachs, 2015; Busby et al., 2017). Switchgrass (*Panicum virgatum* L.), a leading candidate for low-input bioenergy feedstock, exhibits broad phenotypic and genotypic variation that contribute to its ability to tolerate a diverse range of environments (Yang et al., 2009; Casler et al., 2017). However,

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genotypic differences only explain roughly 30% of the variation in cultivar yield responses across different regions, years, and fertilizer rates (Casler et al., 2019). Recent studies suggest that the unexplained variability in cultivar yields and environmental responses may be driven in part by their associations with distinct microbial communities (Rodrigues et al., 2017; Sawyer et al., 2019; Singer et al., 2019a).

Switchgrass cultivars are broadly classified as upland and lowland ecotypes. Lowland ecotypes originate from southern, warm and mesic regions, and upland ecotypes originate from northern, cold and drier regions. Although there are distinct traits across ecotypes, such as earlier flowering and senescence in upland cultivars (Casler, 2012), there is also physiological and phenotypic variation within ecotypes, including in aboveground and belowground traits, drought tolerance, yields, and responses to fertilizer (de Graaff et al., 2013; Aimar et al., 2014; Stahlheber et al., 2020). Multiple recent studies also suggest that switchgrass cultivars belonging to upland and lowland ecotypes have distinct soil microbiomes (Rodrigues et al., 2017; Emery et al., 2018; Revillini et al., 2019; Sawyer et al., 2019; Singer et al., 2019a). However, most previous studies only focused on one or two of the most common cultivars, making it hard to identify general patterns or to determine whether soil microbiomes vary consistently by switchgrass ecotype. Further, with one notable exception (Emery et al. 2018), most studies were conducted on young, immature plants even though switchgrass is a long-lived perennial that reaches stand maturity and peak yields after three years. Given reported ontogenetic differences in plants' microbial communities (Chaparro et al., 2014; Zhalnina et al., 2018), it seems likely that young and mature switchgrass plants will recruit distinct microbiomes that may have different effects on growth or other aspects of plant health such as nutrient acquisition.

Root and soil microbiomes are influenced by plant traits and soil conditions (Fierer, 2017; Saleem et al., 2018). Plants, particularly long-lived perennials, can also alter soil properties which then lead to differences in microbial communities (Liang et al., 2012; DuPont et al., 2014; Zhang et al., 2017). Switchgrass cultivars differ in their root exudate profiles (An et al., 2013), architecture, and tissue chemistry (de Graaff et al., 2013; Stewart et al., 2017)), and these differences may lead to distinct microbiomes. For instance, cultivars with high specific root length (SRL) have a greater relative proportion of thin, high quality (low C:N) roots that provide more labile carbon (C) to microbes (de Graaff et al., 2013; Adkins et al., 2016; Stewart et al., 2017). This influences microbial community C acquisition, soil fungal:bacterial ratios (de Graaff et al., 2013; Roosendaal et al., 2016; Stewart et al., 2017), and the amount of C allocated belowground (Adkins et al., 2016; Stewart et al., 2017). These studies show that differences in root traits and consequent C-provisioning likely contributes to variation in switchgrass cultivar microbiomes, but few studies have measured variation in switchgrass root traits and microbial communities simultaneously (but see Roosendaal et al. 2016; Stewart et al. 2017).

While root traits and soil conditions drive microbial community structure, the strength of these drivers may differ for root- and soil-associated microbial communities (Bulgarelli et al., 2013; Yu and Hochholdinger, 2018). Plant signaling, exudation, and altered abiotic conditions filter and recruit bulk soil microbes to different microhabitats, such as the rhizosphere (soils closely adhering to roots) and endosphere (internal root tissues). Soil-associated microbes are influenced by changes in root exudates and soil conditions, while root microbes are assembled through a two-step process whereby the previously filtered rhizosphere microbes are recruited to the roots through genotype-specific signaling (Bulgarelli et al., 2013). Therefore, although soil conditions affect both root and soil communities, root communities are often a less diverse, but more host-associated subset of the surrounding soil microbes (Bulgarelli et al. 2013). It is also predicted that root-associated communities have greater heritable variation than soil communities (Reinhold-Hurek et al., 2015), but more research is needed to assert this claim. Knowing how microbiomes differ among cultivars' soils and roots as well as what influences microbiome structure will help us understand how microbes may contribute to cultivar- and ecotype-variation in the field and, further, how microbes could be incorporated into switchgrass production.

We hypothesize that root traits and microbial communities will differ among switchgrass cultivars. Further, we expect that a combination of root traits and soil conditions will drive soil microbiome structure, while root microbiome structure will be less diverse, but more distinct among cultivars. We predict that root architectural traits known to increase belowground plant-derived C inputs (e.g., SRL or root diameter) will be an important driver of microbial community structure and biomass. In this study, we address these hypotheses by measuring root traits and microbiomes across 12 mature switchgrass cultivars, asking two primary questions. First, does microbial biomass and community structure vary across switchgrass cultivars? Second, what soil conditions and root traits influence microbial community structure and biomass?

METHODS

SITE DESCRIPTION

We conducted this study in southwest Michigan, USA, at the Great Lake Bioenergy Research Center's Switchgrass Variety Experiment (https://lter.kbs.msu.edu/research/long-term-experiments/glbrc-switchgrass-variety-experiment/) located at the Kellogg Biological Station Long-term Ecological Research Site (42°23′47″ N, 85°22′26″ W). Mean annual precipitation is 100 cm and soils are moderately fertile sandy clay loam (https://lter.kbs.msu.edu/research). In 2009, 12 switchgrass cultivars, including eight upland and four lowland cultivars, were established in a complete randomized block design (four cultivars with poor establishment were replanted in 2010) (Table 1.1 for details on seed source and breeding history). Cultivars were planted at a rate of 9 kg live seed ha^{-1} into 12 plots within four uniformly treated replicate blocks, in the same soil type and within 80 m of one another (n = 48, plots = 4.6 x 12.2 m). The blocks were not irrigated and urea fertilizer was applied annually in the spring (78 kg N ha^{-1}). Pre-emergence weeds were controlled with Quinclorac Drive (1.1 kg ha^{-1}) and Atrazine (0.6 kg ha^{-1}) and post-emergence weeds were treated with herbicides (Glyphosate, 2,4-D, or Dicamba) as needed.

Table 1.1. Details on cultivar origin, sampling date, and establishment year in the common garden experiment. Seed source location and breeding history details from Stahlheber et al. (2020); 'NA' denotes not available.

Cultivar	Ecotype	Sampling date	Establishment year	Breeding history (Native seed source)
Alamo	Lowland	July 27	2009	Seed increase from native remnant prairie[1] (Southern Texas)
EG1101	Lowland	July 13	2010	Improved Alamo-type bred for biomass yield[2] (NA)
EG1102	Lowland	July 27	2010	Improved Kanlow-type bred for biomass yield[2] (NA)
Kanlow	Lowland	July 27	2009	Seed collection from native remnant prairie, selected for leafiness, vigor, late-season greenness[1] (Northern Oklahoma)
Blackwell	Upland	June 28	2009	Seed increase from native remnant prairie[1] (Northern Oklahoma)
Cave-in-Rock	Upland	July 20	2009	Seed increase from native remnant prairie[1] (Southern Illinois)
Dacotah	Upland	June 28	2009	Seed increase from native remnant prairie, selected for leafiness, color and winter hardiness[1] (Southern North Dakota)
EG2101	Upland	July 13	2010	Improved Cave-in-Rock bred for biomass yield[2] (NA)
Nebraska 28	Upland	July 20	2009	Seed increase native remnant prairie[1] (Nebraska)
Shelter	Upland	July 13	2010	Seed increase from native prairie, selected for thick stems, less leafiness, early maturing[1] (West Virginia)
Southlow	Upland	July 20	2009	Seed increase from local remnant native stands to represent local germplasm[3] (Southwest Michigan)
Trailblazer	Upland	July 20	2009	Seed increase from natural grassland, selected for high digestibility and forage[1] (Kansas & Nebraska)

[1]Alderson, J., and W. C. Sharp. 1994. Grass varieties in the United States. USDA, Agriculture Handbook 170. Washington,D.C.
[2]Ceres, Inc. Blade® seeds (www.bladeseeds.com)
[3]Release Brochure for Southlow Michigan Germplasm switchgrass (*Panicum virgatum*). USDA-Natural Resources Conservation Service, Rose Lake Plant Materials Center, East Lansing, MI 48823. Published September 2001, April 2014

SAMPLING AND SOIL ANALYSIS

In June and July 2016, we collected soil cores (2 cm diameter x 20 cm deep) from the rhizome (within 10 cm from the rhizome center) of three randomly chosen switchgrass plants from either end and the center of each block (3 replicate cores x 4 blocks = 12 cores per cultivar). All instruments were sterilized with 70% ethanol in between sampling. Because plant phenological stage can affect microbial communities (Chaparro et al., 2014; Zhalnina et al., 2018) we sampled each cultivar at the same developmental stage – flowering (Emmett et al., 2017). The 12 cultivars flowered over a four-week period and at each sampling date we sampled at least two cultivars (Table 1.1). This controlled for the impact of phenology on microbiome structure, but microbiome differences may have also been affected by variation in host residence time (Dombrowski et al., 2017) or soil conditions. We accounted for some of this temporal variation by including soil moisture content, the edaphic factor that varied most among dates, as a covariate in our analyses (see Analyses section).

After sampling, the soils were stored at 4°C and were frozen at -20°C within 48 hours after sampling. Before freezing the soil cores, we sieved (1 mm) a 30 g subset of the collected soils to remove roots and rocks and subsample for various assays, including chloroform fumigation and potassium sulfate extractions for microbial biomass, soil nitrate and ammonium (12 g soil), volumetric soil moisture content (5 g soils dried at 60°C), and downstream DNA extractions (2 g soil stored at -20°C). Microbial biomass carbon (MBC) and nitrogen (MBN) were analyzed on a TOC analyzer (Shimadzu TOC-VCPH) and calculated by subtracting the total carbon (C) and nitrogen (N) of unfumigated samples from fumigated samples (Vance et al., 1987). Unfumigated potassium sulfate extracts were used to determine soil inorganic ammonium (NH_4^+) and nitrate (NO_3^-) with colorimetric 96-well plate assays. Ammonium concentration was analyzed using ammonia salicylate and ammonia cyanurate as described by (Sinsabaugh et al., 2000). Nitrate reductase enzyme (E.C #1.7.1.1) was used to reduce NO_3^- to NO_2^- and concentrations of NO_2^- were determined using sulfanilamide and *N*-(1-naphthyl)-ethylenediamine. Absorbance for NH_4^+ and NO_3^- assays were read on a Synergy HTX plate reader (BioTek, Winooski, Vermont, USA) at 610 nm and 540 nm, respectfully. All roots collected during initial sieving and remaining soils were stored at -20°C until further root trait analysis and root DNA extractions.

ROOT STERILIZATION AND TRAIT ANALYSIS

The previously frozen sieved roots and undisturbed soils were wet-sieved (2 mm) with nanopure (0.2 µM) water and all visible roots were separated with sterilized tweezers for an average of 30 minutes per sample. These roots were stored at 4°C in nanopure water and scanned within 48 hours. To maintain sterility and minimize microbial cross-contamination, we sterilized all equipment with 70% ethanol in between scans. The roots were scanned (1200 dpi resolution with Epson perfection V600 scanner) in a glass scanning bed with 200 mL nanopure water, exported as tiff files, manually edited to remove image artifacts, and compressed before analyzing root traits with GiA Roots software (Galkovskyi et al., 2012). Following scanning, 0.25 g of the scanned roots (< 2 mm in diameter to standardize for root age) were subsampled and sterilized for root-associated (endophyte) microbial characterization (details below). The remaining roots were weighed and dried at 60°C for one week to calculate the dry:wet root biomass ratio. Predicted total dry root weight was back-calculated using the dry:wet ratio to estimate the dry weight of the 0.25 g subset. This back-calculation of total dry root weight may underestimate actual root weight values if root water content varies with root diameter; an underestimation of root weight could contribute to miscalculations of other root traits, such as mass-weighted specific root length (total root length/dry root biomass). Using GiA Roots, we calculated the following root traits: total root length (cm), average root diameter (cm), total root system volume (cm^3), and specific root length (SRL). SRL was calculated in two ways: 1) mass-weighted SRL which we calculated using the back-calculated dry:wet root ratios (cm total root length/ g total dry root biomass) and 2) volume-weighted SRL (cm total root length/ cm^3 total root volume).

To prepare the root tissues for DNA extractions, we first sterilized the 0.25 g of subsampled roots. Immediately after scanning, we sterilized the subset roots following (Sun et al., 2008): roots were immersed in 70% ethanol for 3 minutes, sterilized with fresh household sodium hypochlorite solution (2.5% available Cl^-) for 5 minutes, rinsed with 70% ethanol for 30 seconds, rinsed ten times with sterile autoclaved water, blotted dry with Kimwipes (Kimberly-Clark, Roswell GA, USA) and frozen at -20°C (Sun et al., 2008). To test root-surface sterilization, the final water rinse was plated on Luria-Bertani agar and incubated at 30°C for 7 days. A majority of the LB plates had bacterial growth after one week of incubation. Although the bacterial growth may suggest incomplete sterilization of the rhizoplane, because these samples were root segments, the cultured bacteria may have been endophytic bacteria that dispersed from the interior of the roots. Due to the thorough sterilization procedure, we believe the remaining microbes are strongly root-associated but cannot conclude they are obligate endophytes. Before DNA extraction, the frozen, surface-sterilized root samples were submerged in liquid N and ground with a tissue lyser (Qiagen Tissue Lyser II, Valencia, California, USA). If any root pieces > 2 mm remained, sterilized scissors (10% bleach and 70% ethanol) were used to more finely cut the roots.

DNA EXTRACTION, SEQUENCING, AND BIOINFORMATICS

DNA was extracted similarly from soil and sterilized roots, but only a subset of cultivars were processed for root-associated microbes. Soil DNA was extracted from 0.25 g of sieved and homogenized sample from all 12 cultivars (n = 144 samples: 12 cultivars x 4 blocks x 3 replicate cores). Root DNA was extracted from approximately 0.25 g of sterilized, ground root tissue from four commonly-planted cultivars (Upland: Cave-in-Rock, Southlow; Lowland: Alamo, Kanlow; n = 48 samples: 4 cultivars x 4 blocks x 3 replicate cores, notated with '+' in all figures). For both soils and roots, we used the MoBio PowerSoil DNA extraction kit and followed all kit-suggested protocols, with an added 10-minute cell lysis step at 65°C before the bead-beating step (MOBIO Laboratories, Carlsbad, California, USA). The purity and quantity of the extracted DNA was examined using a Nanodrop 2000 (Thermo Scientific, USA) and via fluorometry with the Quanti-iT PicoGreen dsDNA kit (Thermo Fisher, USA). We targeted the bacterial V4 region of the 16S rRNA gene (primers 515f/806r) and the fungal ITS1 region (primers ITS1-F/ITS2) for library preparation. Bacterial communities were analyzed for all soil (12 cultivars) and root (4 cultivars) DNA, while fungal communities were only analyzed from the soil DNA (12 cultivars).

Bacterial and fungal PCR and MiSeq Illumina (V2) paired-end sequencing was conducted by the Research Technology Support Facility Genomics Core at Michigan State University (East Lansing, Michigan, USA). Briefly, for both ITS and 16S sequences, reads were assembled, and quality filtered (maxEE < 1.0 and base pairs < 250) using Usearch (version 10.0.240) (Edgar, 2010). Sequences were dereplicated, clustered, chimera checked, filtered de novo, and clustered into unique operational taxonomic units (OTUs) based on 97% identity using the default settings with Usearch UPARSE function. Representative sequences were aligned and classified using the Silva (version 123) and Unite (7.2) reference databases for bacterial and fungal sequences, respectively (Quast et al., 2012; Nilsson et al., 2018). Soil and root-associated bacterial sequences were also aligned to Greengenes (version 13.8) database using Usearch closed-reference (closed_ref) for downstream PICRUSt analysis (DeSantis et al., 2006; Langille et al., 2013). Non-bacterial and non-fungal sequences, singleton OTUs, and samples with poor-sequence coverage were removed from the reference-based OTU tables (Table 1.S1). A bacterial phylogenetic tree was generated using an iterative maximum-likelihood approach with PASTA R package (Mirarab et al., 2015). Phylogenetic-based Weighted Unifrac distance was used for all bacterial community composition analyses. It is challenging to map the variable ITS region to a trustworthy phylogenetic tree (Nilsson et al., 2008), so we used a non-phylogenetic community metric, Bray-Curtis, for the fungal community analyses.

Due to large variation (> 10-fold) in library sizes within and among the root and soil samples, we rarefied our datasets using the “rarefy_even_depth” function in the Phyloseq R package (McMurdie and Holmes, 2014) to control for sequencing depth differences and minimize false discovery rates (Weiss et al., 2017; McKnight et al., 2019). The soil bacterial and fungal datasets for 12 cultivars were filtered and rarefied to 4,694 and 4,153 reads respectively. We compared root and soil bacterial communities for four cultivars on a combined dataset that was rarefied to 2,026 reads. We confirmed that our results were robust to normalization techniques and not biased by rarefaction (McMurdie and Holmes, 2014) by comparing community matrices normalized with rarefaction and Deseq2’s ‘variance stabilizing transformation’ (Love et al., 2014) with a Protest analysis in the Vegan R package (Oksanen et al., 2018). All Protest comparisons were significantly correlated ($p < 0.001$, Table 1.S1) but the combined root and soil dataset had the weakest correlation (r = 0.41) likely due to the 27-fold difference in the sample library sizes. However, because rarefaction is the preferred method for normalizing for large variation in library depth (Weiss et al. 2017), we used the bacterial (Silva-referenced) and fungal (Unite-referenced) rarefied datasets for all community composition and diversity analyses. The rarefied Greengenes-referenced bacterial dataset was used to predict metagenome functions with PICRUSt. Fasta files (NCBI Sequence Read Archive, accession number PRJNA577732) and sequencing pipeline (https://github.com/TaylerUlbrich/SwitchgrassCultivarMicrobiomeStudy) are publicly available.

DATA ANALYSIS: UNIVARIATE STATISTICS

Prior to all data analysis, we assured that all univariate data met assumptions of normality (see supplemental materials for details). Univariate statistics were conducted using one-factor analyses of variance (ANOVA) models and type 3 sum of squares (Satterthwaite's method) with the lm4 and lmerTest packages in R (Bates et al., 2015; Kuznetsova and Brockhoff, P.B., Christensen, 2017). To differentiate the effect of cultivar and ecotype, all variables were analyzed with either cultivar or ecotype as a fixed effect with a random, nested block factor. Since we sampled the cultivars across four weeks to control for phenology-driven variation in microbiomes (Chaparro et al., 2014; Zhalnina et al., 2018), date was confounded with cultivar and ecotype. Due to this collinearity, the model was rank-deficient when both date and cultivar or ecotype were included. Therefore, instead of date, we included soil moisture content, which varied up to 47% across sampling dates (ANOVA, $p < 0.001$; correlation with Julian date $p < 0.001$, r = 0.52), as a covariate when it improved model fit (i.e. lower Akaike information criteria evaluation, AIC). Soil moisture content also correlated with soil nitrate (r = 0.46, $p < 0.002$), which varied by date ($p < 0.001$). However, we decided to include soil moisture content, not soil nitrate, as a covariate because soil moisture content also varied across blocks (ANVOA, $p < 0.001$), allowing us to account for both temporal and spatial heterogeneity. Two extreme outliers that were three times the interquartile range were removed from the soil moisture data, so cultivars EG1102 and Blackwell had only 11 replicates for any model that included soil moisture as a covariate. Several univariate models were improved with soil moisture as a covariate – fungal community richness and evenness, soil and root bacterial richness, microbial biomass nitrogen and carbon, root length – but soil moisture was only a significant predictor variable ($p < 0.05$) for microbial biomass carbon. Post-hoc comparisons (p values adjusted with Benjamini–Hochberg false discovery rate, FDR, $\alpha = 0.05$) were conducted using the multcomp and emmeans R packages (Hothorn et al., 2008; Lenth, 2019). Fungal Shannon diversity and Pielou's evenness did not meet normality assumptions, so we used non-parametric Kruskal-Wallis and Wilcox tests (no block factor included). Pearson correlations were used to determine relationships between edaphic conditions, root traits, and microbial biomass carbon using the 'cor.test' in R (R Core Team, 2018).

DATA ANALYSIS: MICROBIOME COMMUNITY COMPOSITION

Microbial community data were visualized and analyzed using the Vegan, Phyloseq, and ggplot2 R packages (McMurdie and Holmes, 2013; Wickham, 2016; Oksanen et al., 2018). We examined overall variation in the cultivars' microbiome composition using permutation-based ANOVA (PERMANOVA) and betadispersion tests with type 1 sum of squares. PERMANOVAs, betadispersion, and post-hoc pairwise comparisons (FDR-adjusted) were evaluated on the rarefied datasets using the previously described one-factor, blocked model with soil moisture as a covariate with the PRIMER-e software (version 6 & PERMANOVA +, (Anderson et al., 2008). After removing samples with poor sequence coverage and samples with two extreme outliers for the soil moisture covariate, all cultivars had at least 9 replicates for microbiome analyses (Table 1.S2). As in the univariate models, date and cultivar were confounded, so including sampling date in the model did not improve model fit (based on AIC evaluation). However, because the permutational null model can still be calculated for a rank-deficient design, we used supplemental PERMANOVAs with date as a covariate to evaluate the cultivar-level effects when controlling for date. Models with date used instead of soil moisture content were qualitatively similar but the significance was lower (Tables S3, S4). Within sampling date PERMANOVAs were used to further evaluate cultivar-level differences not driven by confounding date effects (e.g., cultivars sampled on the same date in one model, Table 1.1). All ordinations were made with the Phyloseq R package 'ordinate' function with set.seed = 2 for reproducibility (McMurdie and Holmes, 2013).

To further characterize differences in microbial community structure across cultivars, we evaluated the proportion of shared and indicator taxa among the cultivars. We defined shared taxa as those OTUs present in at least 75% of the samples within each cultivar (e.g., 9/12 sample units per cultivar) and across all cultivars. Indicator taxa were identified (after removing singleton OTUs) using the 'multiplatt' function in the indicspecies R package (Caceres and Legendre, 2009) and defined as OTUs present in at least 25% of the samples (3/12 sample units, or indicspecies specificity parameter = 0.25). Rarefied datasets are biased against rare taxa, so it is possible that we identified fewer indicator taxa because less dominant, rare taxa were lost during rarefaction (McMurdie and Holmes, 2014). We also characterized phyla-level differences among cultivars and ecotypes using the 'manyglm' function in the MVAbund R package and ANOVA post-hoc pairwise comparisons (FDR-adjusted) with either cultivar or ecotype as a fixed effect and soil moisture content as a covariate when it improved model fit (based on AIC) (see supplemental materials for details) (Wang et al., 2012; R Core Team, 2018; Ogle et al., 2019).

We were also interested in whether compositional differences based on 16S rRNA were likely to lead to differences in cultivar N-fixation, a function recently identified in switchgrass soils and roots and relevant to cultivar survival in low-nutrient environments (Roley et al., 2018, 2019, 2020). We assessed this by 1) calculating variation in the relative abundance of common N-fixing orders Rhizobiales and Burkholderiales and 2) using PICRUSt to predict the relative proportion of putative N-fixing taxa (Langille et al., 2013) (see supplemental materials for details). Both approaches have limitations but we intended for findings to generate further hypotheses, not to provide definitive assessments of N-fixing potential. The same univariate statistics described above were used to analyze proxies of functional differences among cultivars and ecotypes for the soil- and root-communities.

We further evaluated difference in cultivar microbiomes by determining how edaphic conditions and root traits affect microbiome structure and individual OTU- and order-level abundances. Differences in OTU- and order-level abundance with root traits were evaluated using the 'manyglm' and 'anova' functions in the MVAbund R-package (see supplemental materials for details) (Wang et al., 2012). At the community level, we determined which variables (average root diameter, total root length, soil nitrate, soil ammonium, soil moisture content) significantly contributed ($\alpha = 0.05$) to microbiome structure when controlling for spatial heterogeneity (block) with a partial distance-based redundancy analysis for each dataset: soil bacterial (Weighted Unifrac) and fungal (Bray-Curtis) communities for 12 cultivars and combined root and soil bacterial dataset for 4 cultivars (Weighted Unifrac). We used the 'dbrda' function in Vegan with a conditional matrix for block to determine the relative contribution of block and predictor variables to community structure, as well as the independent, "marginal" effects of each term (Oksanen et al., 2018). Specific root length (volume- and mass-weighted) and total dry root weight were removed from all analyses as they significantly correlated with average root diameter and total root length ($-0.50 < r > 0.50$, $p < 0.05$).

RESULTS

ROOT TRAITS

Total dry root biomass (estimated from dry:wet root calculations), total root length, and mass-weighted SRL (total root length/root biomass) did not significantly differ by cultivar or ecotype ($p > 0.05$, Table 1.S5). Mass- and volume-weighted SRL were significantly correlated ($r = 0.70$, $p < 0.001$), and, unlike mass-weighted SRL, volume-weighted SRL (total root length/root volume) significantly differed among cultivars ($p < 0.01$) but not by ecotype ($p > 0.05$, Figure 1.1A, Table 1.S5).The cultivar differences in volume-weighted SRL were likely driven by average root diameter which significantly differed by cultivar ($p < 0.001$, Figure 1.1B), and was used to calculate root network volume. There was a 30%

difference between the cultivars with the thickest (e.g., Cave-in-Rock and EG2101) and thinnest (e.g., Kanlow and NE28) roots.

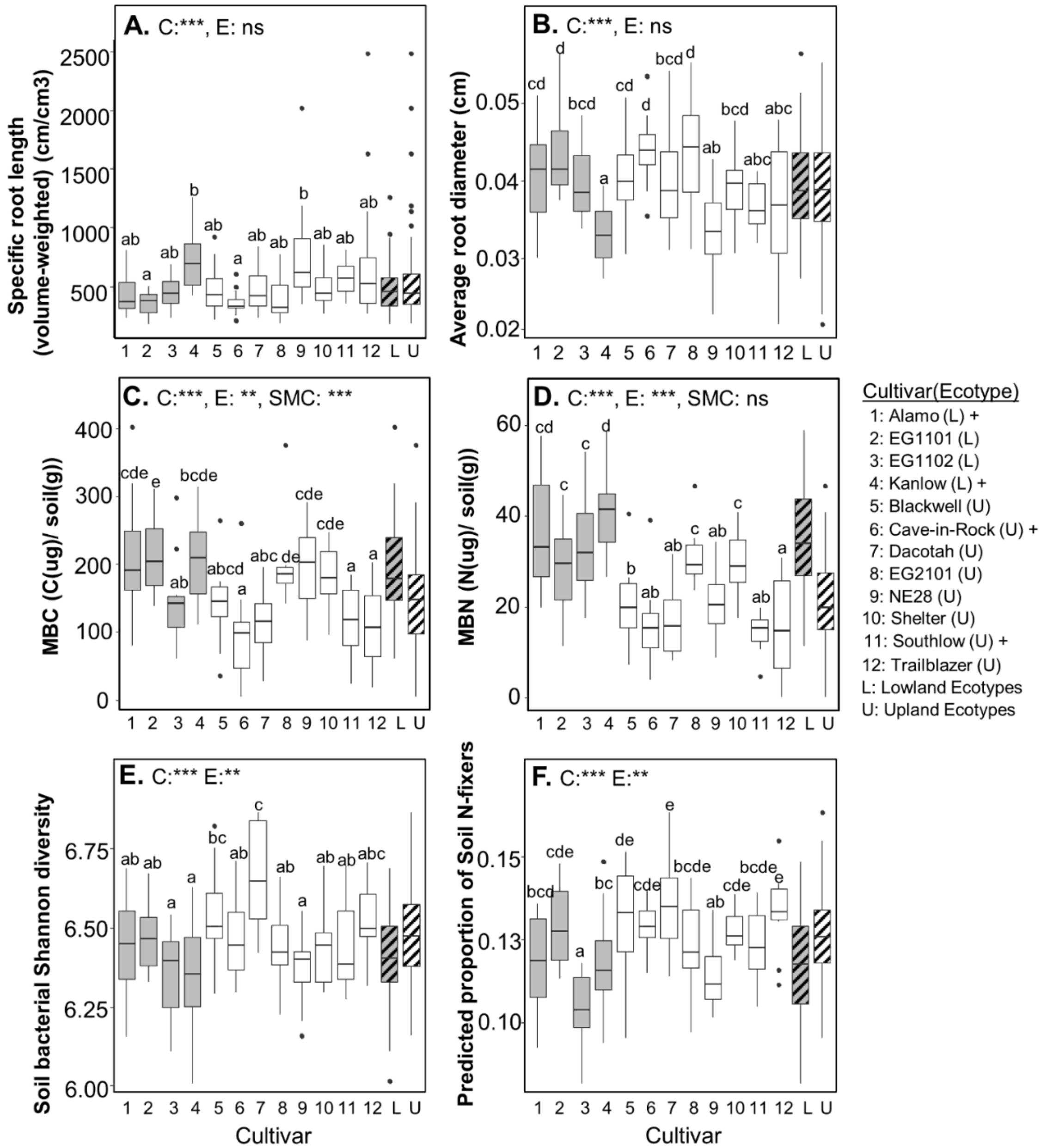

Figure 1.1. Variation in cultivar and ecotype A) volume-weighted specific root length (SRL), B) average root diameter, C) microbial biomass carbon (MBC), D) microbial biomass nitrogen (MBN), E) soil bacterial Shannon diversity, and F) predicted proportion of putative N-fixers in soil. The last two bars represent means for lowland (n = 4; gray boxes) and upland (n = 8; white boxes) ecotypes. Central line is the median value for each cultivar, vertical bars represent the first and third interquartiles of the data, and points are outliers outside the interquartile range. '+' denotes subset of cultivars analyzed for root-associated bacterial communities. Different letters denote significant differences among cultivars (FDR, $p < 0.05$). ANOVA results with fixed cultivar (C) or ecotype (E) term, nested block term and soil moisture content (SMC) included as a covariate when it improved model fit (based on AIC evaluation). Significance values: ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

MICROBIAL BIOMASS

Microbial biomass carbon (MBC) and nitrogen (MBN) significantly differed among cultivars (MBC: $p < 0.001$, MBN: $p < 0.001$) and ecotypes (MBC: $p < 0.01$, MBN: $p < 0.001$) (Figure 1.1C, D), even after controlling for soil moisture content which influenced MBC (soil moisture co-variate with MBC: $p < 0.001$, with MBN: $p > 0.05$) and varied by date ($p < 0.05$). Lowland MBC and MBN were 25% and 65% greater than upland ecotypes, respectively.

SOIL VS. ROOT ASSOCIATED BACTERIAL COMMUNITIES

For a subset of four commonly-planted cultivars (Cave-in-Rock, Southlow, Alamo, Kanlow), we found that root and soil bacterial communities differed in diversity, composition, and the extent to which they were affected by cultivar identity. Microhabitat (soil or root) explained 59% of the overall variance in community composition (Table 1.2, Figure 1.2A), and the root community had five and three times lower bacterial richness and Shannon diversity than the soil communities, respectively (Table 1.S6). The differences in beta diversity between roots and soils were mirrored in their dominant phyla. The most abundant bacterial phyla in the roots (n = 4 cultivars) were Proteobacteria (70%), Actinobacteria (11%) and Bacteroidetes (5%), while the soil communities (n = 4 cultivars) were dominated by Acidobacteria (30%), Proteobacteria (29%), and Verrucomicrobia (11%)(Figure 1.2B). The same phyla were most abundant in the soil communities when analyzed across all 12 cultivars (data not shown). Roots and soils also differed in the relative abundance of common N-fixing orders (Burkholderiales and Rhizobiales), with roots having approximately three times greater relative abundance than soils (Kruskal-Wallis: $p < 0.001$, data not shown).

The degree of cultivar-effect also differed for the root and soil bacterial communities (n = 4 cultivars). Cultivar explained 15% of the variation in the soil community but did not significantly influence the root communities (Table 1.2). The two upland cultivars' soil communities significantly differed from the two lowland cultivars' soil bacterial communities (data not shown), but this may have been driven by differences in soil conditions across sampling dates, which differed for the subset of two ecotypes (Table 1.S4). There was also no cultivar-effect on root or soil bacterial alpha diversity (Table 1.S6) and there were fewer differences in the relative abundance of dominant soil phyla for these four cultivars (Figure 1.4), suggesting that there was less variation among these four commonly-planted cultivars' microbiomes compared to the remaining eight cultivars.

Table 1.2. Percent variability (PERMANOVA R^2) in bacterial community composition explained by habitat (soil or root) and cultivar. Significance values: ns $p > 0.05$, *$p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$. '()' signifies nested factors, '*' signifies the interaction between factors, and 'NA' denotes not applicable for the model.

Factor	Soil & root bacteria (4 cultivars)	Soil bacteria (4 cultivars)	Root bacteria (4 cultivars)
Habitat Effect	%R^2 (p)	%R^2 (p)	%R^2 (p)
Cultivar	2.59 *	15.06**	ns
Block (Cultivar)	6.56*	29.72***	ns
Habitat	58.64***	NA	NA
Cultivar*habitat	ns	NA	NA
Habitat*Block(Cultivar)	6.73*	NA	NA
Soil moisture	ns	4.41*	ns

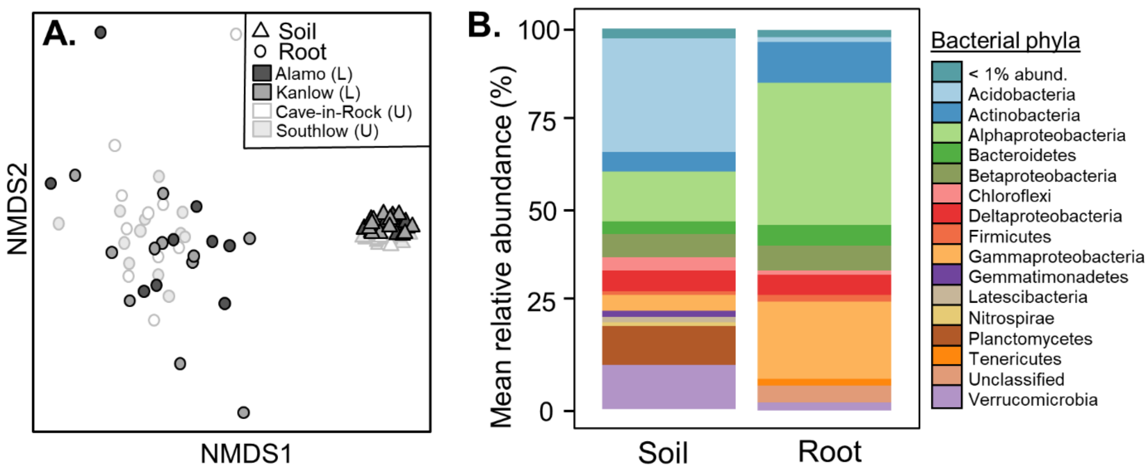

Figure 1.2. A) NMDS ordination of combined soil and root bacterial community (n = 4 cultivars, Weighted Unifrac, stress: 0.08). Soil (triangles) and roots (circles) represent two lowland cultivars (L, dark grey points) and two upland cultivars (U, light grey points). B) Mean relative abundance (%) of bacterial phyla and proteobacteria classes in roots or soils among four cultivars.

SOIL BACTERIAL COMMUNITIES

When evaluated across all 12 cultivars, we found that the soil-associated bacterial communities significantly differed in composition and diversity. Soil bacterial richness, Shannon diversity, and Pielou's phylogenetic evenness differed among cultivars and was 1-3% higher for upland ecotypes for all diversity metrics ($p < 0.05$, Figure 1.1E, Table 1.S7). However, these differences were driven by Dacotah, which had the highest bacterial richness and Shannon diversity (Table 1.S8). Dacotah is a low-yielding upland cultivar that had greater weed invasion which may have contributed to greater bacterial diversity. Even when controlling for sampling date (Table 1.S3) and soil moisture content (Table 1.3), soil bacterial community composition differed among cultivars. When controlling for soil moisture content, block (32%) and cultivar (21%) explained the most variation in community composition, while ecotype only explained 3% of the variation (Figure 1.3A, Table 1.3). The bacterial communities of three cultivars

– Alamo (lowland), EG1102 (lowland), and NE28 (upland) – were more dissimilar from all other cultivars (pairwise comparisons, $p < 0.10$, Table 1.S9). When assessed within sampling date, cultivar explained a significant proportion of variation in the bacterial community composition within one date (16%, $p < 0.05$, Table 1.S10): cultivar NE28 had a significantly different soil bacterial community than the other three upland cultivars (Southlow, Cave-in-Rock, Trailblazer) sampled on the same date.

Table 1.3. Percent variability (PERMANOVA R^2) in microbial community composition explained by cultivar or ecotype. Significance values: ns $p > 0.05$, *$p \leq 0.05$, **$p < 0.01$, *** $p < 0.001$. '()' signifies nested factors and '*' signifies the interaction between factors.

Factor	Soil fungi (12 cultivars)	Soil bacteria (12 cultivars)
Cultivar Effect	%R^2 (p)	%R^2 (p)
Cultivar	11.95*	21.20***
Block (Cultivar)	32.71***	31.94***
Soil moisture	1.85***	3.49***
Ecotype Effect		
Ecotype	1.34*	3.43**
Plot (Ecotype)	43.31***	49.70***
Soil moisture	1.85***	3.49***

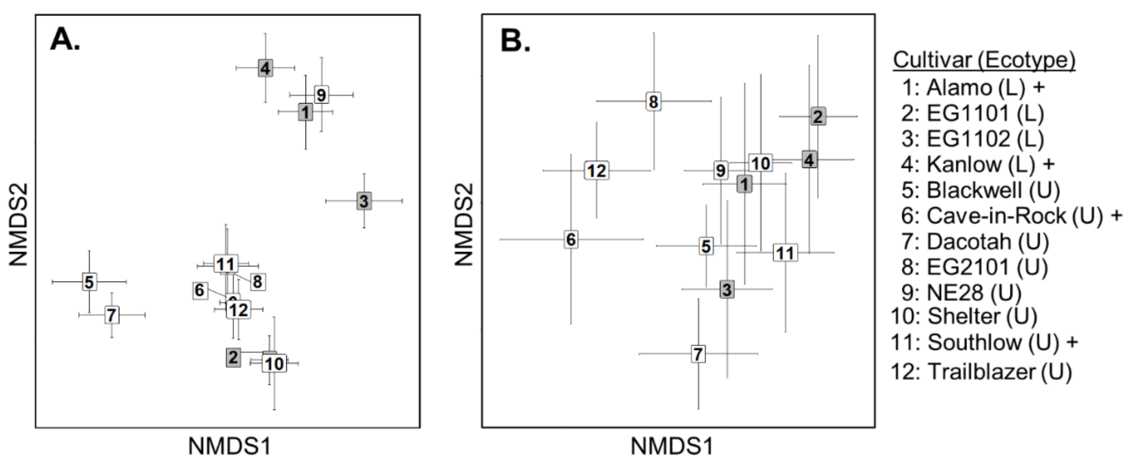

Figure 1.3. NMDS ordination of A) soil bacterial community (Weighted Unifrac, stress: 0.18) and B) soil fungal community (Bray-Curtis, stress: 0.26) across 4 lowland (L, grey points) and 8 upland (U, white points) cultivars. Numbers indicate centroid of sample replicates and horizontal and vertical bars represent ± 1 SE from the centroid. '+' denotes subset of cultivars analyzed for root-associated bacterial communities. See supplemental figure 1.S1 for NMDS with all sample replicates.

The cultivars' soil bacterial communities also differed at the phyla level and are comprised of many shared and few unique taxa. Eight soil bacterial phyla (74.3% of all reads) significantly differed among cultivars (Figure 1.4). Several of these phyla also differed by ecotype; specifically, Bacteroidetes, Planctomycetes, and Verrucomicrobia are more abundant in lowland cultivars, while Actinobacteria, Deltaproteobacteria, and Gemmatimonadetes are more abundant in upland cultivars. At the OTU-level, we found that 160 OTUs (out of 14,590 total) were shared across all cultivars (present in 75% of samples units within and among cultivars). These shared OTUs make up 45% of the total sequences and are dominated by three classes –Acidobacteria (39%), Alphaproteobacteria (17%) and Spartobacteria (12%). In contrast, indicator bacterial OTUs of the 12 cultivars include 683 OTUs and make up 21% of the total sequences dominated by classes Acidobacteria (33%), Alphaproteobacteria (10%) and Deltaproteobacteria (7%).

We used PICRUSt to test whether cultivars' soil and root bacterial communities might have different abilities to fix N_2. We first used NSTI scores to assess whether PICRUSt accurately approximated bacterial function for our sequences. Larger NSTI scores (> 0.15) are expected for highly diverse and largely uncharacterized environments like soils and indicate less phylogenetic relatedness between the predicted OTUs and reference genomes (Langille et al. 2013). The average NSTI scores for the soil samples was 0.23, which is within the typical range for soil samples (Langille et al. 2013) but indicates results should be interpreted with caution due to weak phylogenetic relatedness. Root NTSI (0.32) indicated low relatedness with reference genomes, and therefore were not analyzed. We found that cultivar soil bacterial communities varied in the proportion of OTUs with putative N-fixation genes ($p < 0.001$, Figure 1.1F). On average, upland ecotypes had a greater proportion of predicted soil N-fixers than lowland ecotypes ($p < 0.05$). Predicted soil N-fixer abundance negatively correlated with soil nitrate availability ($r = -0.33$, $p < 0.001$) but did not correlate with soil N-fixation rates ($p > 0.05$) that were measured in a paired study (Roley et al., 2020*,* data not shown). We also compared the relative abundance of common N-fixing orders (Burkholderiales and Rhizobiales) and found no differences among cultivars ($p > 0.05$).

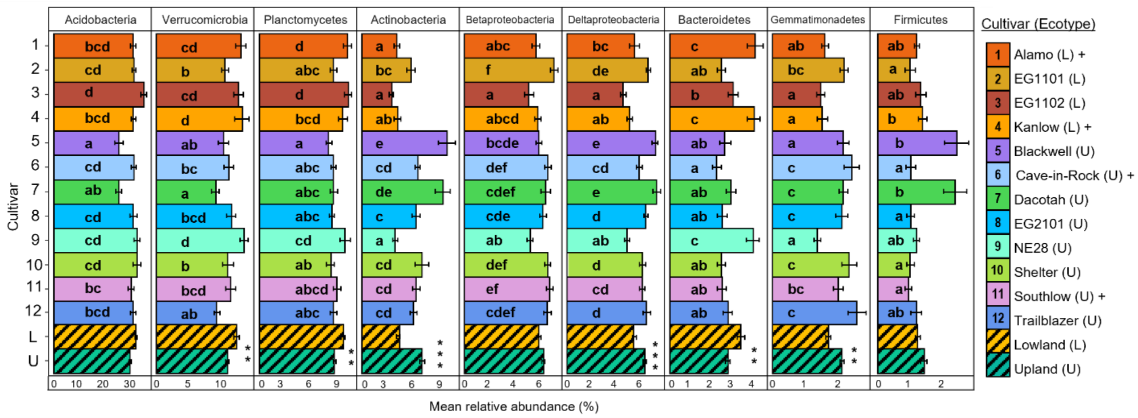

Figure 1.4. Mean relative abundance of bacterial phyla (and proteobacteria classes) that significantly vary among cultivars (MVabund by cultivar: MVabund Dev(11/126) = 1105.8, p = 0.001; each phyla p < 0.05). Bars represent standard error. Phyla are ordered by relative abundance (left = most abundant) and, in each phyla, the bars are ordered by cultivar (1-12), followed by means for lowland (L; n = 4) and upland (U; n = 8) ecotypes. '+' denotes subset of cultivars analyzed for root-associated bacterial communities; '*' above ecotypes indicate statistically significant differences among ecotypes (ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

SOIL FUNGAL COMMUNITIES

When controlling for soil moisture content, the primary drivers of soil fungal community composition were similar to the bacterial community: block explained the most variation (33%), followed by cultivar (12%) and ecotype (1%) (Table 1.3, Figure 1.3B). However, unlike the bacterial communities, the cultivar-level effects on fungal communities were not robust to variation across (Table 1.S3) or within sampling dates (Table 1.S10). Fungal community diversity (richness, Shannon, evenness) also did not differ by cultivar or ecotype ($p > 0.05$, Table 1.S7).

Only one fungal phylum, Rozellomycota, significantly differed in abundance among the cultivars (MVabund 9, $p < 0.01$), and no phyla differed by ecotype (MVabund, $p > 0.05$). OTUs identified as Rozellomycota only made up 0.73% of the reads, and therefore likely did not contribute much to variation in cultivar microbiomes. The dominant fungal phyla were Ascomycota (32%), Basidiomycota (17%), Mortierellomycota (14%) and Glomeromycota (9%), but 25% of the fungal OTUs were unclassifiable at phyla level. Among fungal OTUs (4,064 total), 37 were shared across all cultivars (present in 75% of samples units within and among cultivars). These shared OTUs made up 35% of the total sequences and were dominated by classes Mortierellomycetes (28%), Sordariomycetes (23%), and those Unclassified (29%). Indicator fungal OTUS of the 12 cultivars make up 25% of the total fungal sequences and include 213 OTUs dominated by classes Sordariomycetes (19%), Dothideomycetes (17%), and 27% were unclassified at class level.

EFFECT OF EDAPHIC PROPERTIES AND ROOT TRAITS ON MICROBIOME

To further understand variation in cultivar microbiomes, we investigated how root traits and edaphic conditions (N and water content) impact community structure. Across all 12 cultivars, the five predictor variables (average root diameter, root length, soil moisture content, soil nitrate, soil ammonium) explained more variation for the soil bacterial (10%) than the soil fungal (5%) communities (Table 1.4). Mirroring the PERMAONVA results, spatial heterogeneity (conditional block variance) explained a significant portion of community dissimilarity for the soil bacteria and fungi. While controlling for variance due to spatial heterogeneity, variance in the bacterial community structure was most explained by soil nitrate (6%) and soil moisture content (2%) while the fungal community was most explained by soil nitrate (1%) and root length (1%). Within the four cultivars evaluated for soil and root bacterial community composition, nitrate explained 6% of the variation in the soil community, but no edaphic conditions or root traits contributed to variation in the root communities (Table 1.4).

We also investigated whether the relative abundance of bacteria or fungal taxa (at the order- and OTU-level) or microbial biomass correlated with root traits (average root diameter, root length). We did not identify any bacterial orders that correlated with root traits, but identified one fungal order, Mortierellales, that negatively correlated with root length (MVabund $p < 0.05$, correlation: r = -0.41, $p < 0.001$). Further, microbial biomass carbon negatively correlated with root length (r = -0.23, $p < 0.01$) but not with average root diameter ($p > 0.05$).

Table 1.4. Percent variability (R^2) of microbiome structure explained by soil conditions and root traits using db-RDA analysis. Percent explained partitioned by conditional (block), constrained (all predictor variables), and unconstrained (residuals) factors; ns $p > 0.05$, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$. 'NA' denotes not-applicable for models that were not significant ($p > 0.05$).

	Soil bacteria (12 cultivars)	Soil fungi (12 cultivars)	Soil bacteria (4 cultivars)	Root bacteria (4 cultivars)
Nitrate (µg N/ g dry soil g)	6.36***	1.17**	5.72**	NA
Ammonium (µg N/ g dry soil)	ns	ns	ns	NA
Soil Moisture Content (g/g dry soil)	1.86**	ns	ns	NA
Average Root Diameter (cm)	ns	ns	ns	NA
Root Length (cm)	ns	1.06*	ns	NA
Model significance	***	***	**	ns
Conditional Variance	7.67	6.23	9.83	NA
Constrained Variance	10.12	5.03	15.31	NA
Unconstrained Variance	82.22	88.75	74.86	NA

DISCUSSION

We examined bacterial and fungal microbiomes, soil variables, and root traits across 12 mature switchgrass cultivars grown in a common garden experiment. Overall, we found that cultivars vary in their average root diameter, have different soil microbial biomass, and associate with distinct soil, but not root, bacterial communities. Differences in the soil microbiomes were driven by variation in root traits, phenology, and soil properties, and were more pronounced at the cultivar level than across ecotypes. Still, cultivar was a weaker driver of soil communities than among-plot soil heterogeneity, and we saw less overall variation in fungal communities. These subtle but significant differences in root traits and soil bacterial communities that we observed may contribute to variation in cultivar yields, environmental responses, or ability to provide beneficial ecosystem services (e.g., soil C sequestration).

CULTIVARS HAVE A GREATER EFFECT ON SOIL BACTERIAL THAN ROOT BACTERIAL OR SOIL FUNGAL COMMUNITIES

Traditionally, ecotypes are used to classify differences among switchgrass cultivars, but we found greater differences in switchgrass microbiomes across cultivars than between ecotypes. We found that cultivar explained 10-20% of the variance in soil microbiome beta diversity, while ecotype explained less than 5% of the variation; these stronger cultivar effects were also found in a previous study on switchgrass cultivar soil bacterial and fungal communities (Singer et al., 2019a, 2019b), but Emery et al. (2018) observed no cultivar effects on arbuscular mycorrhizal fungi (AMF) in the same common garden experiment. Our findings show that at this site, the weak effect of cultivar on AMF is true for a broader assessment of fungi as well (assessed via the ITS region). Despite overall weak effects of ecotype on OTU-level composition, ecotypes differed in the relative abundance of several dominant bacterial phyla. This may suggest that higher-level taxonomic differences are conserved across ecotypes, while finer, OTU-level differences occur among cultivars. Although we did not examine specific functions in this study, OTU-level differences among cultivars could contribute to variation in their nutrient cycling or yields. In fact, in the same common garden experiment, Stahlheber et al. (2020) found that aboveground traits and yields varied more among cultivars than between ecotypes, a pattern that could have been influenced by microbiome differences.

On a subset of four cultivars, we predicted that there would be a greater cultivar-effect on root-associated than soil bacterial communities, but in fact the soil bacterial communities differed more among cultivars. The weak cultivar-effect on the root communities could have been influenced by our cultivar selection, such that the other eight cultivars – which had greater variation in soil communities – may have also had more distinct root microbiomes. Further, it is also possible that we under-sampled the root bacterial diversity, as many chloroplast and mitochondrial sequences reduced microbiome sampling. Despite these potential caveats, other studies conducted on a similar number of cultivars also report greater cultivar-level differences among soil than root microbiomes in switchgrass (Singer et al. 2019a, n = 4 cultivars) and rice (Edwards et al., 2015a); therefore, we posit that our observation of greater cultivar-effects on soil than root communities is biologically relevant. The soil communities also had less within cultivar variation than the root communities. This has been observed previously (Edwards et al. 2015) and may suggest that there is greater intraspecific variation in traits that affect microbial recruitment to the rhizosphere (e.g., root structure, exudation, or diffuse signaling) than in traits that regulate microbial entry into the root (e.g., physical and immune system interactions). In fact, it may be that plant traits associated with root microbiome assembly are conserved at even higher taxonomic levels, as Singer et al. (2019b) found that two *Panicum* species have similar endophyte bacterial communities. The role of genotype on microbiome structure remains unclear, but it could be clarified with surveys of microbiome variation across multiple genotypes and species. Additionally, it seems that the proximity of the microbiome to the plant may not be a good predictor of the influence of plant genotype on microbiome structure, but finer-scale sampling (e.g. soil, rhizosphere, rhizoplane, and endosphere) would help confirm this (e.g., (Edwards et al., 2015a).

EDAPHIC CONDITIONS AND PLANT TRAITS INFLUENCE SOIL COMMUNITY STRUCTURE

Soil water and nitrogen content influenced switchgrass cultivar soil, but not root microbiomes, while root traits only affected the soil fungal community. Soil nitrate availability explained the most variation in the cultivars' soil microbiomes, but no edaphic or root traits influenced the root community composition. Similar patterns were observed by Singer et al. (2019b) – *Panicum* species' rhizosphere soil communities were more affected by soil type than endosphere communities. These edaphic conditions are considered to have larger effects on soil microbiomes than plant identity (Fierer, 2017), but the observed differences in soil N in this study could be driven by the cultivars' differential effects on N cycling (Roley et al., 2020) which could in turn influence the microbiome (Revillini et al., 2019). Contrary to our prediction, we did not observe any effect of root traits on bacterial community structure, but found that fungal community structure was affected by root length. Root length may be a particularly important trait for root colonizing-fungi (e.g., AMF), since root system size determines the amount of niche space available for colonization. Few studies simultaneously evaluate fungal community structure and root length, but in the same common garden experiment, AMF root colonization correlated with root biomass (Emery et al., 2018). Our results supports this finding because root length significantly correlated with root biomass ($r = 0.75$, $p < 0.001$). In these conclusions we are presuming that root traits drive bacterial and fungal communities, but the observed correlation could also describe microbes driving root traits (Verbon and Liberman, 2016; Petipas et al., 2020).

We found that spatial variability (block factor) also explained a surprisingly large percent (> 30%) of variation in the soil microbiomes. Although our blocks were the same soil type and within 80 m of one another, they differed in soil moisture and nitrogen content (also in paired study, Roley et al. 2020). Our analysis of microbiome composition and edaphic conditions controlled for this block effect, yet it is difficult to disentangle the relative contribution of cultivar traits, spatial heterogeneity, and sampling date on these edaphic conditions and, in turn, microbiome structure. Further, it is possible that the variation across blocks contributed to greater plasticity in the cultivars' traits, thus making it more challenging to identify correlations between traits and microbiome structure. Overall, although the primary drivers of switchgrass microbiome structure are challenging to disentangle, our results suggest that heterogeneous soil conditions, plant traits, and feedbacks between plant traits and soil conditions all likely contribute to microbiome variability among switchgrass cultivars.

The strength of relationships between root traits and soil microbiomes can also be influenced by soil fertility and sampling techniques. Our study was conducted on productive, annually fertilized soils, and cultivar differences and plant-microbe associations may be stronger in less-fertile, marginal soils, when plants and microbes are more dependent on one another (Bell et al., 2014; Sawyer et al., 2017). Sawyer et al. (2017) found that switchgrass cultivar microbiomes were more distinct in less fertile soils. It is also possible that cultivars that were grown outside of their native range (e.g. not from the north-central United States) had weaker effects on their microbiomes because they could not associate with their native, potentially co-evolved microbial communities. Studies of cultivars in common gardens across many sites could elucidate the contribution of native range or seed source on plant-microbial interactions. Further, because we did not sample the soils directly adhering to the roots or use primers to target root-colonizing microbes (e.g., AMF) we may not have captured the microbes most influenced by root traits and exudates. Finally, we found that cultivars vary in average root diameter and, therefore, soils beneath each cultivar likely differ in the amount of root turnover and development. Microbial composition and function has been shown to vary with root age, type (e.g., seminal or nodal root), and location (e.g., root branch or tip(Marschner and Baumann, 2003; de Graaff et al., 2013; Kawasaki et al., 2016), but sampling with soil cores made it challenging to identify the effects of root age, type, or location on soil microbial communities. Therefore, future studies should use methods that standardize root age (e.g., use of root-in-growth cores) or root type and location (e.g., visualizing root differences and sampling within rhizoboxes) to better understand how root traits influence microbiome structure (Yu et al., 2018).

Plant developmental stage (e.g., phenology, maturity) also contributes to microbiome variability (Edwards et al., 2018; Zhalnina et al., 2018; Na et al., 2019). We sampled cultivars at the same stage (flowering) to control for this variation, but sampling on different dates may have increased differences in edaphic conditions that influence the microbiome. Yet, when we controlled for variation among sampling dates, cultivar still contributed to variation in the soil bacterial, but not fungal communities. This suggests that the fungal communities were more influenced by variation in abiotic conditions across dates, or that cultivars with different phenology and, thus, sampling dates, had more dissimilar fungal communities. In contrast, bacterial community structure was more strongly influenced by cultivar identity, which explained a significant percent (16%) of the variation in bacterial community structure within one of the four sampling dates. We hypothesize that greater differences were not observed within the other three sampling dates because cultivars with comparable phenology (e.g., flowering at the same time) likely have other similar traits and, thus, more similar microbial communities than cultivars with different phenology. However, to better understand the effect of similar phenology and traits on cultivar microbiomes, future studies should evaluate the switchgrass cultivar microbiomes across multiple phenological stages (e.g., Wagner et al., 2016; Qiao et al., 2017; Na et al., 2019) as both the microbiome structure and the magnitude of cultivar effects may change with phenological stage (Inceoglu et al., 2010; Na et al., 2019).

FUNCTIONAL IMPLICATIONS AND CONCLUSIONS

Differences in cultivar root traits and microbial biomass could contribute to variability in the cultivars' soil C-cycling and C sequestration potential. We found differences in microbial biomass and root diameter, but not root biomass, across cultivars. Another study conducted in the same common garden experiment, however, did find differences in root biomass among cultivars (Emery et al. 2018). These differences in average root diameter have the potential to drive variation in the cultivars' C-cycling and microbial community structure. Root systems with high SRL, corresponding to long, thin roots, positively correlate with switchgrass-derived soil C (Adkins et al., 2016; Stewart et al., 2017), decomposition (de Graaff et al., 2013, 2014), bacterial:fungal ratios (de Graaff et al. 2013), and microbial biomass (PLFA-C) (Stewart et al. 2017). Greater rhizodeposition from thin roots can directly contribute to soil C pools, as well as indirectly influence soil C by supporting the growth and turnover of microbial communities which, in turn, contributes to greater soil C and aggregate stability (Grandy and Neff, 2008; Tiemann et al., 2015). Therefore, the cultivars we identified with thinner roots (Kanlow and NE28) or with higher microbial biomass C (many lowland cultivars) may have greater potential to increase soil C in marginal soils and improve C sequestration.

The observed differences in microbial communities and root traits could also influence cultivar nutrient cycling and tolerance to different environmental conditions, in turn, affecting yield. We found that the predicted N-fixer abundance in soil communities varied among cultivars and ecotypes. A paired study (same location and sampling dates) found that the rate of soil N-fixation also varies among cultivars (Roley et al. 2020), but our PICRUSt-inferred functional potentials did not correlate to the measured rates (data not shown). Still, our results suggest that functional differences are likely, and future studies should investigate N-fixation and other functions with more targeted approaches, as microbiome function may influence the suitability of various cultivars for surviving under different soil conditions.

In summary, we found that root traits, microbial biomass, and soil bacterial community composition differs among switchgrass cultivars, and that this variation could contribute to differences in their potential as bioenergy crops. Despite ecotype being the most common way to group cultivars, soil microbiome structure and root traits differed more among cultivars than ecotype. Future research on switchgrass-microbe interactions should examine multiple cultivars rather than relying on results from one model cultivar to make ecotype-level assumptions. Understanding how cultivar traits influence microbial communities can improve our ability to select and breed cultivars with optimal microbiome-mediated traits, like high N-fixation or C sequestration. We also observed larger cultivar effects on bacterial than fungal soil communities, suggesting that there may be greater heritable variation and, thus breeding potential, for switchgrass bacterial than fungal microbiomes. This study shows that differences in switchgrass cultivars that have been documented aboveground also exist belowground and have the potential to influence the future success and ecosystem service provisioning of switchgrass as a bioenergy crop.

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CHAPTER TWO: PLANT ROOT EXUDATES AND RHIZOSPHERE BACTERIAL COMMUNITIES SHIFT WITH NEIGHBOR CONTEXT[2]

ABSTRACT

A plant's neighborhood context can alter its interactions with other organisms, but little is known about how these dynamics occur belowground, especially with soil microbes. Microbial communities in rhizosphere soil are influenced by many factors, including abiotic conditions and root-derived signals. In particular, root exudates have strong effects on rhizosphere assembly, respond to changes in abiotic conditions, and help plants interact with neighbors. Therefore, we predicted that root exudates likely play a central role in neighbor-induced shifts in rhizosphere communities. We conducted a greenhouse experiment to test this and determine how the rhizosphere bacterial community of a focal plant, *Panicum virgatum*, changed when beside different neighbors, and whether these shifts were mediated by neighbor-induced changes in root exudation. We found that neighbor altered both focal plant exudates and rhizosphere community, and that changes were largest when the focal plant was beside the most competitive neighbor, *Rudbeckia hirta*, which reduced both focal plant growth and nitrogen uptake. Several factors contributed to neighbor impacts on rhizosphere assembly, including neighbor-induced changes in root exudates during nitrogen-limitation and microbial spillover from roots of larger neighbors. Using an additional soil incubation, we also found that these changes in exudates can have even greater effects on soil nutrients than on microbial assembly. Overall, we show that neighbors influence one another's microbiomes, and highlight neighbor-induced changes in root exudates as one mechanism through which this may occur. This work suggests that rhizosphere assembly may differ in mixed-species communities and thus emphasizes a need for microbiome studies that consider neighborhood context.

INTRODUCTION

Decades of research show that a plant's neighbors can alter its interactions with other organisms. This ecological concept, referred to as "associational effects", has been primarily studied in aboveground, plant-insect interactions (Barbosa et al., 2009; Underwood et al., 2014). However, increasing evidence suggests that plants' belowground neighborhoods are also important (Li et al., 2016; Huang et al., 2018; Kong et al., 2018; Chen et al., 2019). Similar mechanisms that drive aboveground

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associational effects may also occur belowground, including direct effects of neighbor plants on local abiotic and biotic conditions, changes in plant physiology and chemistry, and interplant signaling. For instance, root exudates transmitted between neighbors can serve as warning signals that stimulate herbivore defenses in a focal plant (Glinwood et al., 2003; Babikova et al., 2013). Still, despite increasing recognition of the role of neighborhood contexts, little is known about their broader role in shaping plants' interactions with soil microbial communities (Howard et al., 2021).

One challenge with identifying the role of neighbors on soil microbiomes is that many studies either focus on isolated plants in pots or, in the case of field studies, ignore neighborhood context. Still, these controlled studies show that plants assemble a species-specific soil microbiome near their roots, the rhizosphere (Berg and Smalla, 2009; Philippot et al., 2013). Plants also host species-specific rhizosphere communities in field settings (Rosenzweig et al., 2013; Schöps et al., 2020), but in some cases rhizosphere structure changes with neighborhood richness (Bakker et al., 2013; LeBlanc et al., 2015). Therefore, it is unclear if the same mechanisms that mediate rhizosphere assembly in isolated plants are maintained in diverse plant communities. In fact, in many cases, predictions of plant community dynamics fail if they are based on greenhouse studies with isolated plants (Forero et al., 2019), perhaps because the predictions assume that rhizosphere communities do not change with active neighbor interactions.

Only a few studies have investigated how neighborhood context alters rhizosphere assembly (Hausmann and Hawkes, 2009; Bakker et al., 2013; Morris et al., 2013; Hortal et al., 2017a; Cavalieri et al., 2020; Mony et al., 2021), and the patterns and mechanisms driving these associational effects vary widely. In some cases, plant neighbors have neutral effects on each other's rhizospheres, such that they maintain their species-specific communities while growing together (Hausmann and Hawkes, 2009). Other times, a focal plant's rhizosphere may begin to resemble that of its neighbor (Hawkes et al., 2006; Hortal et al., 2017a). This can occur during strong neighbor competition for nutrients (Hortal et al., 2017a), especially if the neighbor roots are overlapping, as occurs in dense grasslands (Vieira et al., 2019). Dense, overlapping root systems may affect rhizosphere assembly through 'microbial spillover', whereby microbes from a larger root system disperse to its neighbor through close root contact. Because rhizosphere assembly is related to host phylogeny (Emmett et al., 2017), it is likely that spillover of novel microbes from a distantly related neighbor would drive greater shifts in a focal plant rhizosphere (Mony et al., 2021). Still, more competitive neighbors do not always overwhelm a focal plant's rhizosphere assembly (Cavalieri et al., 2020). Together, these studies show that diverse neighborhoods affect host rhizosphere assembly through many mechanisms, but that these mechanisms may vary by plant species or other factors, and need to be elucidated.

Neighbor-induced shifts in root-derived metabolites, root exudates, may play a particularly important role in belowground associational effects. Root exudates have been shown to recruit particular microbes to a plant's rhizosphere (De-la-Peña et al., 2008; Zhalnina et al., 2018). In addition to their role in rhizosphere assembly, root exudates also change in response to neighbors (Badri et al., 2012; Herz et al., 2018; Kong et al., 2018; Weinhold et al., 2022). Abiotic conditions have similarly strong effects on root exudates. Water and nutrient limitation, each of which may occur with a competitive neighbor, have strong effects on root exudate profiles (Dakora and Phillips, 2017; Gargallo-Garriga et al., 2018; Smercina et al., 2020). Therefore, because root exudates respond to neighbors and environmental cues, and are central to rhizosphere assembly, we hypothesize that neighbor-induced shifts in root exudates also contribute to belowground associational effects.

Here, we use a greenhouse experiment to investigate how plant neighbors alter rhizosphere dynamics. We first hypothesize that the focal plant's rhizosphere bacterial community composition will change when beside different neighbors, consistent with a belowground associational effect. Second, we hypothesize that the bacterial community changes will correlate with neighbor-induced shifts in root exudate profiles. If this is true, we would expect to see an overall correlation between the exudate composition and bacterial communities, as well as repeatable shifts in taxa with the manipulation of key exudates. Third, we hypothesize that strongly competitive neighbors will induce larger shifts in focal plant exudates and rhizosphere bacteria than less competitive neighbors. This is because strong competitors are more likely to induce a physiological response in the focal plant and may also cause greater spillover effects if their root systems are larger.

METHODS

STUDY SPECIES

The focal species (*Panicum virgatum* L. *v*ar. Southlow) is a C4, perennial grass native to tallgrass prairies and is also a candidate bioenergy crop (McLaughlin and Kszos, 2005). *P. virgatum* is suggested to associate with beneficial microbial communities that can improve its growth and tolerance of stressful conditions (Hestrin et al., 2021). However, it is unknown if these microbial associations change with shifts in *P. virgatum*'s growing context, for instance if it grows in diverse prairies or monocultures for bioenergy. To this end, we studied the effect of neighborhood interactions on *P. virgatum's* rhizosphere community and root exudates. The neighbor species included three perennial prairie species known to co-occur with *P. virgatum*, including a *Andropogon gerardii* Vitman (C4 grass), *Koeleria macrantha* Ledeb. (C3 grass), and *Rudbeckia hirta* L. (forb).

GREENHOUSE EXPERIMENT

We carried out a greenhouse experiment at Michigan State University's W.K. Kellogg Biological Station. The focal plant, *P. virgatum,* was exposed to five neighbor treatments: either no neighbor, or a single neighbor conspecific, *A. gerardii, R. hirta,* or *K. macrantha*. Each plant species was also planted in 'monoculture treatments', consisting of each plant neighbored by its conspecific (two plants per pot). Each treatment was replicated five times, with a total of 45 pots (9 treatments x 5 replicates = 45 total pots, see Figure 2.S1 for experimental design).

All seeds were sterilized and grown for five weeks in flats with a light layer of soil inocula before transplanting into the experimental treatments (see supplemental for more details on seedling preparation). After five weeks, we removed the seedlings, rinsed the roots with RO water, and planted them into their neighborhood treatments. The plants were grown in 3.8 L pots (Elite Nursery Classic 300) with a substrate mixture of autoclaved sand, autoclaved vermiculite and field soil inocula (45:45:10). This substrate will be referred to as soil throughout the text, though we recognize that it is an artificial soil mixture. The soil inocula, which was the same used in the germination flats, was collected from a nearby mid-successional grassland at the W.K. Kellogg Biological Station Long-Term Ecological Research Site in southwest Michigan. The soil is a sandy-loam and the field was dominated by *Bromus* sp. grasses. The soil was sieved (4 mm) and kept at 4 °C for ten weeks prior to inoculating the pots. Although the original field soil community likely changed after ten weeks, the purpose of the inoculum was to provide an initial soil community that was not pre-conditioned by any species in the study, not to represent the original field community.

The plants grew for eight weeks in their neighbor treatments with temperatures controlled at a maximum of 26 °C during the day and minimum of 15 °C at night with 14 hours of artificial lighting. They were watered with RO water as needed and fertilized twice with ammonium nitrate (equivalent of 46 kg N ha^{-1} pot^{-1}) in 200 mL of half-strength Hoagland's solution (2.5 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM KH_2PO_4, 1.0 mM $MgSO_4$, 0.024 mM H_3BO_3, 0.004 mM $MnCl_2 \cdot 4H_2O$, 0.102 µM $CuSO_4 \cdot 5H_2O$, 0.382 µM $ZnSO_4 \cdot 7H_2O$, 0.248 µM $Na_2MoO_4 \cdot 2H_2O$, 5.4 µM NaFeEDTA).

PLANT HARVEST AND SOIL ANALYSES

After eight weeks of growth, we collected plant biomass, rhizosphere soil, homogenized pot-level bulk soil, and focal plant root exudates. Rhizosphere was collected by carefully removing each plant, untangling their roots, and collecting the closely-adhering soil that remained after multiple shakes; the rhizosphere soils were stored at -20 °C for DNA extractions. Though the rhizosphere soil was attached to the focal roots, the fact that the neighbor and focal roots were intertwined likely led to greater microbial spillover that we could not distinguish from neighborhood effects (see Discussion). All non-focal plants' roots and shoots were dried (55 °C) while the focal plants were left intact and temporarily placed in sterile whirlpacks (Nasco, USA) with 0.05 mM calcium chloride buffer solution prior to root exudate collection (details below). The remaining bulk soil from the pots was homogenized for pot-level nutrient and microbial biomass analyses. Briefly, bulk soils were stored at 4 °C and subsampled for gravimetric soil moisture content analysis (55 °C), and chloroform fumigation and potassium sulfate extractions for microbial biomass and soil nitrate and ammonium analyses (see supplemental for more details on soil analyses).

EXUDATE COLLECTION

We collected root exudates from the focal plants (n = 25) using a soil-hydroponic-hybrid method (Oburger and Jones, 2018). The benefit of this method is that plants are grown in soil-like conditions with active microbial communities so that exudates are not altered by artificial sterile conditions, but a minor drawback is potential artifacts from root damage during washing (Williams et al., 2021a), as well as microbial excretion and consumption of root exudates during collection. Despite these caveats, this approach is still a common method used to study interactions between root exudates and rhizosphere communities (Vieira et al., 2019; Brisson et al., 2021). After harvesting, the focal plants were placed in buffer solution (0.05 mM calcium chloride, $CaCl_2$), left to recover for three to six hours, and then the roots were cleaned of residual soil and detached, dead roots. Submerging the roots in fresh solutions prior to exudate collection can help remove metabolites released from damaged tissues (Oburger and Jones, 2018), but a recent study suggests that a recovery period of at least three days is preferred (Williams et al., 2021a). Once all root systems were cleaned, we collected root exudates by submerging the intact plants in flasks with 250 mL of fresh 0.05 mM $CaCl_2$ solution. Flasks were covered with parafilm to reduce airborne contamination and kept in the dark with a foil covering. The flasks, including three no plant controls, were placed on a shaker table with supplemental lights, and the exudate solutions were filter sterilized (0.2 µm) and frozen at -80 °C after six hours (17 hr to 23 hr). Over the six hour collection period, it is possible that some metabolites were degraded or consumed by root microbes, as a collection time of less than four hours is often recommended in non-sterile systems (Oburger and Jones, 2018; Williams et al., 2021a). The focal plant shoots and roots were dried at 60 °C, weighed, ground (Qiagen Tissue Lyser II), and analyzed for total C and N (Costech Elemental Combustion System 4010).

EXUDATE ANALYSIS

We thawed and subset the frozen root exudate solutions into 50 mL centrifuge tubes for downstream exudate analysis, including quantification of dissolved organic carbon (DOC) and metabolite fingerprint analysis with liquid- and gas-chromatography mass-spectrometry (LC-MS, GC-MS). Thawed exudate solutions were run on a Total Organic Carbon (TOC) analyzer to determine total DOC per sample (Shimadzu TOC-VCPH); two samples were missing from this analysis because there was not enough excess exudate solution. Another 50 mL subset of extracts were lyophilized and sent for LC- and GC-MS analysis. During lyophilization, several tubes cracked, so the initial exudate volumes vary between samples and, therefore, normalized metabolite data are reported.

Lyophilized exudates were prepared for MS analysis by resuspending them in 2 mL of methanol:water (80:20). Tubes were centrifuged and extracts were transferred into 2 mL glass vials, dried down with a centrifugal vacuum evaporator, and resuspended into a final volume of 300 µL. Untargeted LC-MS analyses were performed directly on the extracts. For GC-MS measurements, an aliquot of 200 µL from each exudate sample was dried down into an HPLC vial and chemically derivatized to trimethylsilyl ester before analyses (Kim et al., 2005). The LC-MS and GC-MS files were processed using MZmine 2.37 (Pluskal et al., 2010) and Metabolite Detector 2.5 (Hiller et al., 2009), respectively. LC-MS features were identified using exact mass and retention time from an in-house library of metabolites, corresponding to the second-level of putative identification (Sumner et al., 2007). GC-MS metabolites were identified using a modified version of FiehnLib (Kind et al., 2009) and verified using NIST14 GCMS library. LC- and GC-MS datasets were combined, filtered, and metabolic features within each sample were normalized by the total intensity of chromatograms (details on MS analysis and data filtering described in supplemental methods). Missing data (NAs) were imputed for downstream statistical analyses using the 'MissForest' R package (Stekhoven and Bühlmann, 2012).

SOIL INCUBATION EXPERIMENT

Due to limitations with correlating omics datasets (Pang et al., 2021; Zancarini et al., 2021), an additional soil incubation was performed to establish a more causal link between changes in root exudates and rhizosphere assembly. We manipulated the relative concentration of malic acid in soil incubations to determine if the bacteria enriched while neighboring *R. hirta* were driven by greater malic acid exudation. Malic acid was exuded more when the focal plant neighbored *R. hirta* (see Results, Figure 2.S5), and it was also the second most abundant metabolite in the exudate solutions, and contributed to shifts in overall bacterial community structure (see Results, Figure 2.4B).

We added two exudate solutions (100 µg C g^{-1} dry soil) – high malic acid (75% malic acid, 8.33% citric acid, 8.33% sucrose, 8.33% glucose) and low malic acid (25% malic acid, 25% citric acid, 25% sucrose, 25% glucose)— along with a water control to soil mesocosms daily over 24 days. Before additions, the exudate solutions were brought to a neutral pH (6.0) with potassium hydroxide (pH probe: Mettler-Toledo, Five Easy Plus), filter sterilized (0.22 µM), divided into weekly aliquots, frozen, and thawed for weekly additions.

The soil mesocosms (237 mL mason jar) were filled with the equivalent of 30 g dry soil and raised to 65% water holding capacity (WHC) with autoclaved milli-Q water (0.22 µm). The soils were collected from the same mid-successional grassland used for the greenhouse experiment, albeit two years later, sieved (4 mm) and analyzed for WHC. The jars were wrapped with Breathe-Easy® (Sigma-Aldrich) micropore film to allow gas exchange but prevent airborne contamination, maintained at 55% WHC, and stored in the dark at room temperature (approximately 25 °C). After 24 days, the soils were subsampled for DNA extractions, gravimetric soil moisture content , and chloroform fumigation and potassium sulfate extractions for soil DOC, total extractable nitrogen (TN), and microbial biomass analyses, following the same procedures detailed previously.

DNA EXTRACTION, ILLUMINA SEQUENCING, AND BIOINFORMATICS ANALYSIS

DNA was extracted from 0.25 g of homogenized soil from the initial soil inocula, greenhouse experiment soils (focal, neighbor plants, and no-plant controls, n = 80) and soil incubation soils (n = 41) using the MoBio PowerSoil DNA extraction kit (MOBIO Laboratories, Carlsbad, CA, USA). We targeted the bacterial V4 region of the 16S rRNA gene (primers 515f/806r) with MiSeq Illumina (V2) paired-end sequencing, conducted by the Research Technology Support Facility Genomics Core at Michigan State University, East Lansing, Michigan. The reads were quality filtered and clustered into unique operational taxonomic units (OTUs) based on 97% identity using the Silva (version 123) bacterial database at 80% confidence (Quast et al., 2012), and a bacterial phylogenetic tree was created using an iterative maximum-likelihood approach with the 'PASTA' R package (Mirarab et al., 2015).

The library sizes significantly differed by 2.5-fold among greenhouse treatments (all greenhouse & focal samples ANOVA $F_{9,73} = 3.81$, $p < 0.001$); therefore, we rarefied both the greenhouse and incubation dataset to 16,224 reads. To reduce the effect of rare or spurious taxa, we removed any OTUs not present in at least 10 samples, resulting in 6,221 taxa for the greenhouse dataset and 4,234 in the soil incubation dataset. All bacterial beta- and alpha-diversity metrics were calculated on the rarefied and filtered datasets. See Supplemental Information for more details on sequencing and bioinformatics methods.

UNIVARIATE DATA ANALYSIS

For either experiment, plant and soil characteristics, microbial biomass, and bacterial alpha diversity data were confirmed to meet normality assumptions and analyzed using one-factor analyses of variance (ANOVA) and type 3 sum of squares (Satterthwaite's method), followed by post-hoc pairwise comparisons (Benjamini-Hochberg False Discovery Rate, FDR, α = 0.05(Lenth, 2019). Data that did not meet normality assumptions were transformed (soil nitrate, square-root transformed). For the greenhouse experiment, individual one-way ANOVAs were used to determine the effect of treatment on either the focal plant, monoculture, or pot-level responses. Microbial biomass and soil chemistry data were collected and analyzed at the pot-level, representing the shared conditions for both plants in the pot.

We conducted additional ANOVAs with focal plant aboveground biomass as a covariate to account for differences driven by neighbor competition. While the reduction of a focal plant's biomass is a classic definition of competition (Grace, 1995) we also calculated the relative strength of competition using RII for an alternative assessment of neighbor competition (Armas et al., 2004). We paired the five replicates for each treatment for the calculation. Negative RII values indicate that the focal plant is suppressed by its neighbor through competition, with a more negative value indicating stronger competition, while positive values indicate facilitation.

MULTIVARIATE DATA ANALYSIS

Multivariate analyses of the bacterial composition were performed on Weighted-Unifrac distance matrices from the rarefied community and all multivariate analyses of the exudate data were performed on Euclidean distance matrices. Because Weighted Unifrac analyses can bias against rare, less-abundant taxa, we further partitioned the focal plants' bacterial communities to determine if dominant or non-dominant taxa were driving the treatment effects. We defined 'dominant' as the top 10% most abundant taxa across all focal plant samples and the non-dominant taxa as the remaining 90%. The dominant group included 571 taxa and made up 69.6% of the focal plant bacterial reads, while the non-dominant taxa included 5,142 taxa and made up 30.4% of the focal plant bacterial reads.

We evaluated the effect of plant treatment on the bacterial communities and exudate profiles using one-factor permutational multivariate ANOVA tests (PERMANOVAs, n = 9999 permutations), followed by post-hoc pairwise comparisons with FDR adjustment (α = 0.05). Additional PERMANOVAs with focal plant biomass included as a covariate were used to control for the effect of neighbor plant competition. We identified bacterial genera representative of each treatment in the greenhouse and soil incubation experiments using indicator species and differential abundance analyses. The magnitude and direction of neighborhood effects on rhizosphere assembly was assessed with PERMANOVAs that compared the community structure of the focal plant with that of its direct plant neighbor, its neighbor species' monoculture, and the focal plant *P*. *virgatum* monoculture. For this analysis, the OTU abundance of the monoculture plant treatments were averaged into a single value using 'merge_samples' (fun = "mean") in the 'Phyloseq' R package (McMurdie and Holmes, 2013).

To further partition variation in the focal plant root exudates, we used sparse Partial Least Squares Discriminant Analysis (sPLS-DA) to determine which of the identified exudates contributed to the greatest variation in treatments. We then determined how the top ten identified exudates correlated to the soil and plant characteristics using p-adjusted Pearson correlations. We used variance partitioning analysis to identify which of the plant and soil variables had the largest effect on the complete root exudate profiles. We identified three extreme outliers (three times the interquartile range) in the gravimetric soil moisture content data, so these samples were removed from analyses that correlated soil conditions with microbial or exudate data.

Finally, we investigated the relationship between the bacterial and exudate datasets. Because no single correlation technique yields the same result (Weiss et al., 2016), especially when comparing two -omics datasets (Pang et al., 2021), we used multiple statistical approaches to determine if neighbor-induced shifts in the root exudates were correlated with shifts in the bacterial community. First, we performed a principal component analysis on the exudate dataset and Hellinger-transformed bacterial dataset and then evaluated the similarity in the matrices with a Protest analysis. Second, we used variance partitioning analysis to determine how the top ten most abundant identified exudates, neighbor treatment (categorical), and focal plant C:N affected bacterial community composition. Third, we evaluated the effect of the top ten identified exudates, as well as the focal soil and plant characteristics, on bacterial community structure using distance-based redundancy analysis (dbRDA).

Lastly, we looked for specific relationships between bacterial genera and the 140 identified exudates using the 'CCREPE' (Compositionality corrected by renormalization and permutation package) R package (Schwager et al., 2020). This method outperforms traditional correlation techniques, such as Pearson and Spearman, which are not suitable for compositional data and are known to have high false positive rates for compositional data (Pang et al., 2021).

The statistical program R (version 4.0.5) was used for all analyses and all package and parameter information is detailed in Supplemental Table 2.S7. Sequencing pipeline and code are available at https://github.com/TaylerUlbrich/NeighID_Switchgrass; raw sequence fastq files can be found on the NCBI repository (Accession number PRJNA773254).

RESULTS

NEIGHBOR IDENTITY ALTERED FOCAL PLANT BIOMASS AND SOIL PROPERTIES

Overall, plant neighbor altered focal plant biomass, root and shoot C:N, and soil conditions (Table 2.1, Figure 2.1). These properties were most affected when *R. hirta* was a neighbor. *R.* hirta decreased total switchgrass biomass by 70%, was larger than other neighbors (Figure 2.S2A, aboveground biomass: $F_{3,16} = 78.39$, $p < 0.001$; belowground biomass: $F_{3,16} = 16.70$, $p < 0.001$), and was classified as the strongest competitor by the relative strength of competition index ($p = 0.064$, Table 2.1, Figure 2.S2B). *R. hirta* maintained this large size in a monoculture, where it had 2.2 times greater total biomass than all other species in monoculture (Table 2.1).

In addition to plant biomass, neighbor effect on focal plant C:N and soil moisture was also most prominent with *R. hirta* (Table 2.1, Figure 2.1B and Figure 2.S3A). Focal plants neighbored by *R. hirta* had 66 and 65% higher root and shoot C:N (respectively) than focal plants in the other neighbor treatments (Figure 2.1B). *R. hirta* also used more moisture, as *R. hirta* pots had lower soil moisture when present as a neighbor, and in monoculture (Table 2.1, Figure 2.S3A). Neighbor treatment had a small effect on soil nitrate (Table 2.1, Figure 2.S3B), though in monocultures, *K. macrantha* had four times greater soil nitrate than any other plant monoculture (Table 2.1). There was also clear uptake of soil nitrate during plant growth, as the no plant control had 8.5 times higher soil nitrate than all other treatments.

Table 2.1. Effect of neighbor treatment on focal plant growth, soil conditions, bacterial community, and root exudates, as well as differences among monoculture treatments in greenhouse experiment. ANOVA and PERMANOVA results shown; PERMANOVAs conducted on bacterial community structure (Weighted Unifrac) and root exudate profiles (Euclidean); significant p values bolded ($p < 0.05$).

	Focal Treatments (Focal plant)		Monoculture Treatments	
	F	*p*	F	*p*
Plant and Soil Variables				
Total Biomass (g)	4.01	0.015	23.54	<0.001
Aboveground biomass (g)	4.65	0.008	21.17	<0.001
Belowground biomass (g)	2.15	0.112	10.73	<0.001
Shoot C:N	10.95	<0.001	NA	NA
Root C:N	6.13	0.002	NA	NA
Soil Moisture (g water g^{-1} dry soil)	6.99	0.001	4.35	0.020
Soil nitrate (µg NO_3^- g^{-1} dry soil)	3.61	0.023	10.76	<0.001
Rii (aboveground biomass)	3.09	0.057	NA	NA
Rii (belowground biomass)	0.53	0.667	NA	NA
Rii (total biomass)	2.02	0.151	NA	NA
Bacterial Community and Exudates				
Shannon Diversity	6.16	0.002	2.99	0.044
Pileau's Evenness	0.79	0.546	1.46	0.24
Chao1	9.96	<0.001	3.55	0.024
Microbial biomass carbon	2.19	0.107	2.61	0.087
Bacterial community structure (all taxa)	1.35	0.056; $R^2 = 0.21$	5.40	<0.001; $R^2 = 0.31$
Bacterial community structure (dominant taxa)	1.29	0.099; $R^2 = 0.21$	NA	NA
Bacterial community structure (non-dominant taxa)	1.32	0.014; $R^2 = 0.21$	NA	NA
Root Exudates (all)	4.92	<0.001; $R^2 = 0.50$	NA	NA

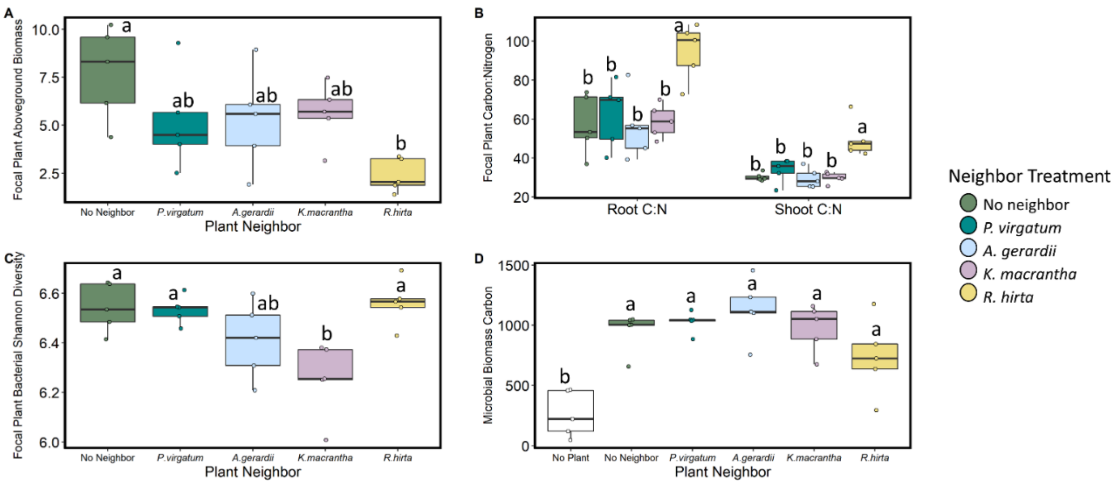

Figure 2.1. Neighbor effect on focal plant A) aboveground biomass, B) shoot and root carbon:nitrogen content, C) bacterial Shannon diversity, and D) microbial biomass carbon (collected at the pot-level). The central line is the median value, vertical bars represent the first and third quartile, and dots represent individual replicate values. Different letters denote significant differences among treatments (false discovery rate, $p < 0.05$).

THE MOST COMPETITIVE NEIGHBOR HAD STRONGER EFFECTS ON FOCAL PLANT BACTERIAL COMMUNITY

The most dominant rhizosphere phyla across all plant species were Proteobacteria, Acidobacteria, Verrucomicrobia, Plantomycetes, and Bacteriodetes (37%, 15%, 13%, 9%, 8% relative abundance respectively). Each of the four plant species were associated with differently structured, but not sized, bacterial communities (Table 2.1). Diversity also differed and was highest in the *P. virgatum* monoculture and lowest in the *K. macrantha* monoculture (Table 2.1).

Neighbor identity did not affect the amount of microbial biomass, but significantly altered both the diversity and structure of the focal plant's bacterial community (Table 2.1, Figure 2.1C & 1D). Bacterial Shannon and Chao1 diversity, but not evenness, differed by neighbor treatment; specifically, diversity was lower when switchgrass neighbored *K. macrantha.* These patterns did not change when we controlled for the effect of competition on focal plant aboveground biomass (Table 2.S1). Neighbor also affected community structure, explaining 20% of the variation in the focal plant's bacterial community (Table 2.1), and these shifts were more influenced by the non-dominant taxa than the dominant taxa (PERMANOVA $p = 0.014$ for non-dominant taxa; PERMANOVA $p = 0.099$ for dominant taxa; Table 2.1).

The neighbor-induced shifts in microbial community structure were strongest with *R. hirta*, which was also the most competitive neighbor (see above). Still, when we controlled for neighbor competition (by including focal plant biomass as a covariate), the focal plants' non-dominant taxa differed by neighbor treatment (Table 2.S1 & 2). Compared to the other neighbor treatments, *R. hirta* led to twice as many indicator genera (n = 15) in the focal plant rhizosphere, including *Sphingomicrobium, Zymomonas, Methylotenera, Caulobacter, Methylophilus, Flavobacterium* (Table 2.S3 for complete list of indicator genera). Interestingly, the relative abundances of these genera were also greater in the *R. hirta* monoculture compared to the other neighbor monocultures (Figure 2.S4).

We further evaluated how neighbors altered the focal rhizosphere by comparing the focal and neighbor bacterial communities to those in their monocultures (Figure 2.2). *A. gerardii* and the focal species (*P.virgatum*) rhizosphere communities were similar to one another when each was grown in monoculture, and they did not alter each other's rhizosphere communities when grown together in a neighborhood, for both dominant and non-dominant communities (Figure 2.2A & 2D). *K. macrantha* and the focal species, on the other hand, had more dissimilar rhizosphere communities, but when in a shared neighborhood, their communities resembled the focal monoculture (Figure 2.2B & 2E). These patterns observed with the *A. gerardii* and *K. macrantha* neighbors did not differ for the dominant versus non-dominant taxa, but they did with *R. hirta.* When sharing a pot with *R. hirta,* the dominant taxa resembled that of the *R. hirta* monoculture (Figure 2.2C), but the non-dominant taxa rhizospheres were distinct from either monoculture species (Figure 2.2F). These comparisons also revealed that despite the distinct effect of each neighbor on the focal rhizosphere, there was strong homogenization between the interacting plants: the focal rhizosphere communities did not significantly differ from that of their direct neighbor in a shared pot (PERMANOVA effect of treatment by plant position: Weighted Unifrac; treatment effect: $F_{3,39} = 2.21$ $p < 0.001$, $R^2 = 0.16$; shared pot effect: $F_{1,39} = 0.63$, $p = 0.91$; treatment*pot $F_{3,39} = 0.58$, $p = 0.99$).

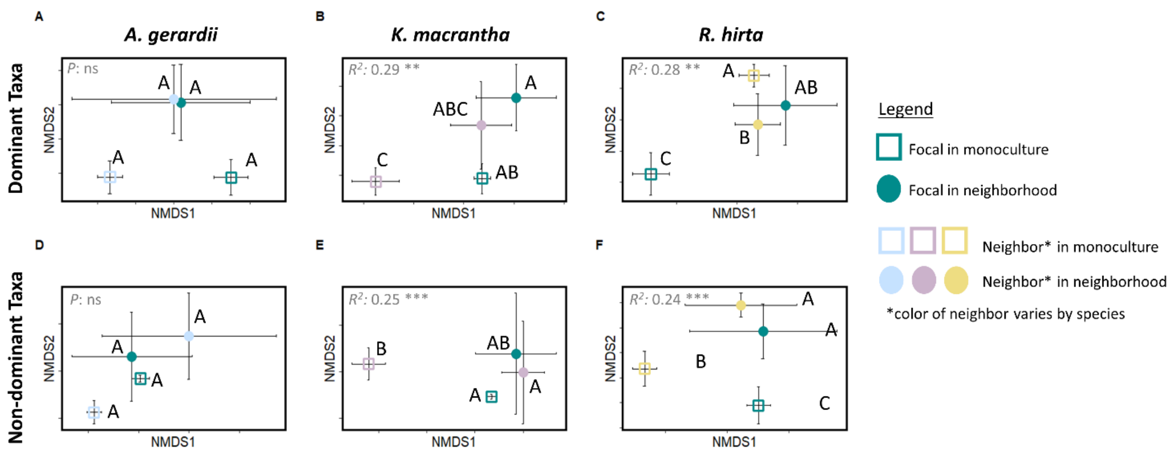

Figure 2.2. Nonmetric multidimensional scaling (NMDS) ordination comparing focal (*P. virgatum*) and neighbor plant bacterial communities in shared pot neighborhoods to their respective monoculture treatments (OTU abundance averaged at pot-level). A-C represent dominant taxa (top 10% most abundant) and D-F represent non-dominant taxa (lower 90% abundant). Open squares represent monocultures for either focal plant (dark blue) or neighbor species (*A. gerardii* – light blue, *K. macrantha* – purple, *R. hirta* – yellow); closed circles represent the focal or neighbor species in a shared pot neighborhood. Each centroid is the average of sample replicates (n = 5) and bars indicate ± 1 standard error from the centroid. PERMANOVA results presented in top left of each panel (R^2, ns $p > 0.05$, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$); Different letters denote significant differences among treatments (false discovery rate, $p < 0.05$).

NEIGHBOR IDENTITY ALTERS FOCAL PLANT ROOT EXUDATES

We detected 14,648 unique metabolite features from the root exudates of the focal *P. virgatum* plants (LC-MS and GC-MS), of which 140 of them were putatively identified. The top 10 most abundant identified compounds from exudates samples were quinate, malic acid, qluconic acid, hydropxypyruvate, myo-inositol, fructose, lyxose, shikimic acid, and azelaic acid (metabolite abundance by treatment - Figure 2.S5). Of all unknown and identified exudates, quinate and malic acid were the top two most abundant.

Neighbor identity had a strong effect on the focal plant root exudate profile, but not on the amount of total carbon exuded (total organic carbon: $F_{1,18} = 1.12$, $p = 0.379$). This effect was even stronger than the effect of neighbor on rhizosphere community structure, as neighbor treatment explained 50% of the variation in the focal plant root exudates (Table 2.1) and 37% when we controlled for variation in focal aboveground biomass (Table 2.S1). When neighbored by *R. hirta* and conspecific *P. virgatum* the exudates were most dissimilar from the no neighbor treatment (pairwise *p-values* Table 2.S2).

Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) further indicated that the *R. hirta* and *K. macrantha* neighbor treatments had the most distinct exudate profiles (Figure 2.3A). The first component (C1) of the sPLS-DA explained 15% of the variation and separated the *R. hirta* neighbor treatment from the other four treatments. Of the top 15 discriminant exudates for C1, eight of them were most abundant in the *R. hirta* treatment, including fumaric acid, malic acid, stearic acid, cytosine, 1-methylguanosine, hypoxanthine, and sn-glycerol-3-phosphate (Figure 2.3B). Component two (C2) explained 14% of the variation and separated the *K. macrantha* treatment, which had a greater abundance for 12 of the top 15 discriminant exudates for C2 (Figure 2.3C).

We used variance partitioning to determine which factors had the largest effect on the root exudate profiles. Neighbor treatment explained the most variation in the exudate profiles (41.3%), followed by the focal plant's aboveground biomass (16.5%), root C:N ratio (9%) and soil moisture content (6.0%). All four variables cumulatively explained 51.5% of the variation in root exudates. The top ten most abundant identified exudates also correlated with these plant and soil factors (Figure 2.S6A). Malic acid was exuded significantly more with the *R. hirta* neighbor (14-fold greater peak area with *R. hirta,* Figure 2.3B & Figure 2.S5) and had the strongest correlations with these factors (Figure 2.S6A, but the correlations were driven by the significantly lower C:N plant tissue and soil moisture in the *R. hirta* focal treatment (Figure 2.S6B-E).

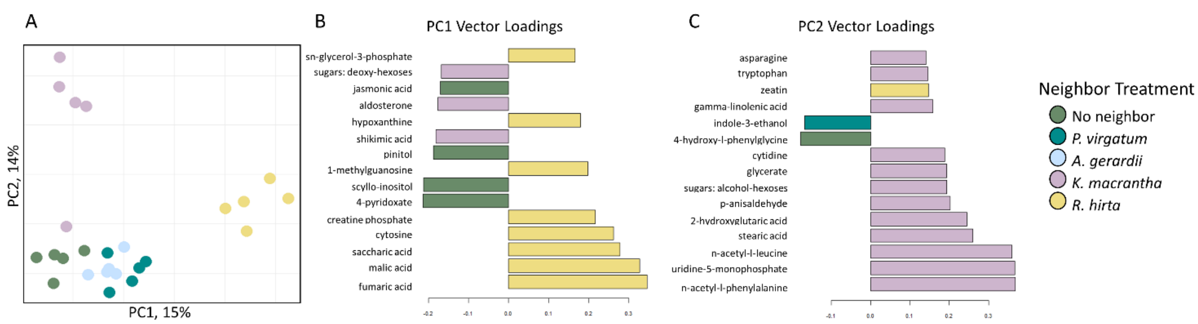

Figure 2.3. A) Principal Coordinates Analysis (PCA) of focal plant identified exudates by treatment. Loading vectors from sparse Partial Least Squares Discriminant Analysis (plsDA) for Component 1 (B) and Component 2 (C). Bar colors in B & C indicate which treatment had the highest mean value for each exudate.

EXUDATES AND BACTERIAL COMMUNITIES ARE CORRELATED AT WHOLE COMMUNITY-LEVEL

Overall, we found that the focal plant root exudates had stronger correlations with the entire bacterial community than with individual genera. Protest analyses showed that the significant and strong correlation observed with the whole community (Procrustes Protest test on PCA axes, r = 0.78, m2 = 0.47, p = 0.006), was similar for both the dominant and non-dominant taxa (Dominant taxa: Protest test, r = 0.80, m2 = 0.45, p = 0.009; Non-dominant: Protest test, r = 0.71, m2 = 0.54, p < 0.001). The variance partitioning (Figure 2.4A) and db-RDA results (Figure 2.4B) suggested that the top ten most abundant exudates, specifically malic acid (dbRDA: $R^2 = 0.08$, $p = 0.021$) and stearic acid (dbRDA: $R^2 = 0.06$, $p = 0.113$), drove shifts in the community, and that focal shoot C:N (dbRDA: $R^2 = 0.06$, $p = 0.090$) also played a role. Similar to the Protest results, these two dominant exudates influenced both the dominant and non-dominant taxa, but only the dominant taxa were impacted by focal shoot C:N (db-RDA analyses, Table 2.S4). Despite significant correlations at the whole community level, we identified only six significant correlations between individual exudates and bacterial genera. The bacterial genera *Methylophilus* had the most significant correlations with exudates, including malic acid, fumaric acid, and pyruvic aldehyde (*nc correlation metric* > 0.50, *p.adj* < 0.02) (Table 2.S5 for complete list).

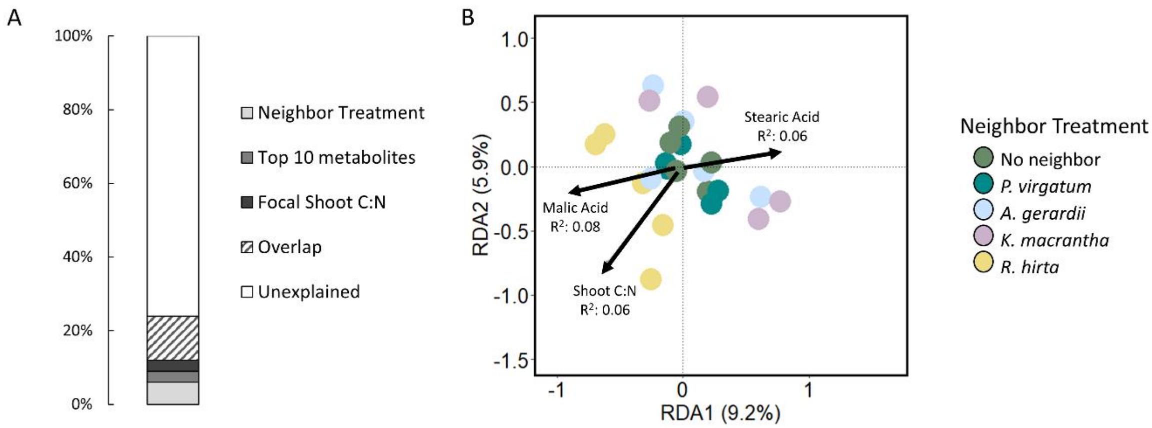

Figure 2.4. Variance in the focal plant bacterial community composition (Weighted-Unifrac) explained by exudates, plant and soil factors. A) Variance partitioning analysis depicting the proportions of variance in bacterial community explained by the neighbor treatment (6%), top then most abundant identified metabolites (3%), and focal shoot C:N (3%). All factors explain 11% of the total variation in bacterial community. B) Distance-based Redundancy Analysis of focal plant rhizosphere bacterial communities by neighbor treatment; arrows indicate environmental variables and identified exudates explaining significant variation in the community structure ($p \leq 0.10$). These three variables account for 20% of the variation in the community (constrained proportion); overall dbRDA model statistics: ANOVA $F_{3,20} = 1.64$, $p = 0.008$. Only four replicates shown for *K. macrantha* treatment after removing an extreme outlier from soil moisture data.

MALIC ACID ALTERS BACTERIAL COMMUNITIES AND INCREASES SOIL CARBON AND NITROGEN

Malic acid was exuded more near *R. hirta* (Figure 2.3B and Figure 2.S5) and had significant impact on the overall community structure (Figure 2.4B), but not on particular bacterial genera. Therefore, we used a soil incubation experiment to more causally link malic acid exudation with shifts in bacterial community structure. We found that the concentration of malic acid has a strong effect on bacterial community structure (PERMANOVA: $F_{3,17} = 3.83$, $p = 0.04$, $R^2 = 0.32$), with *Brevundimonas, Pedobacter, Pseudoxanthomonas, Pseudospirillum, Hirschia, Flavitalea, Chryseolinea* genera enriched in the high malic acid treatment (Table 2.S6 for complete list). However, these same genera did not strongly correlate with malic acid in the greenhouse experiment and, similarly, were not enriched when the focal plant neighbored *R. hirta* (Figure 2.S7).

The malic acid additions also altered soil carbon, nitrogen, and microbial biomass. The high malic acid soils had two times greater dissolved organic carbon ($p < 0.001$, Figure 2.5A) and extractable total nitrogen ($p < 0.001$, Figure 2.5B) than the low malic acid soils. Microbial biomass C:N ratio was 55% lower in the high malic acid soils ($p = 0.011$), and this was reflected in the microbial biomass N, which was 18% greater in the high malic acid soils ($p = 0.035$, Figure 2.5D) while microbial biomass C was marginally lower in the high malic acid soil ($p = 0.087$, Figure 2.5C).

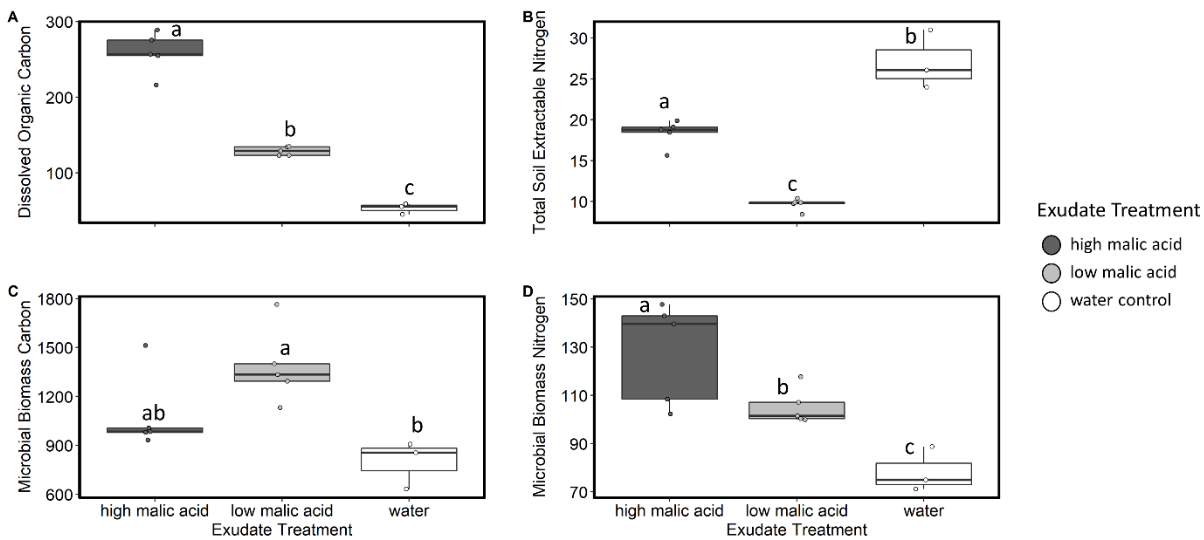

Figure 2.5. Effect of malic acid concentration on soil incubation A) dissolved organic carbon, B) total soil extractable nitrogen, C) microbial biomass carbon, and D) microbial biomass nitrogen. The central line is the median value, vertical bars represent the first and third quartile, and dots represent individual replicate values. Different letters denote significant differences among treatments (false discovery rate, $p < 0.05$).

DISCUSSION

NEIGHBORS INDUCED SPECIES-SPECIFIC CHANGES IN FOCAL PLANT RHIZOSPHERE BACTERIAL COMMUNITY

We found that neighbor identity altered the composition of the focal plant rhizosphere community, offering support for our first hypothesis that associational effects, widely observed aboveground, also occur in soil. The functionally-similar C4 grass neighbor*, A. gerardii*, had a neutral effect on the focal rhizosphere, while the forb neighbor, *R. hirta*, altered both the focal plant's dominant and rare bacterial taxa. These neighbor-specific effects on soil communities have been observed in previous studies (Hausmann and Hawkes, 2009; Mony et al., 2021), and could have several explanations. First, a more functionally-dissimilar neighbor, like *R. hirta* in our study, may introduce novel taxa to a focal plant's existing microbiome. This was also previously observed with mycorrhizal fungal communities (Mony et al. 2021). Differences in growth and competition for resources can also alter the strength of associational effects. This was likely a strong contributing factor in our study, as *R. hirta* was the fastest growing neighbor plant, both in monoculture and neighborhood. *R. hirta's* root system was larger than all other neighbor species, which likely led to greater microbial spillover to the focal rhizosphere (discussed more below). Overall, we cannot distinguish the effect of neighbor growth and competition for resources from their functional dissimilarity, but future studies might experimentally investigate what predicts the strength of belowground associational effects, and why.

CHANGES IN ROOT EXUDATES CORRELATE WITH OVERALL BACTERIAL COMMUNITY BUT NOT INDIVIDUAL GENERA

Overall root exudate and bacterial community profiles were strongly correlated, suggesting that neighbor-induced changes in exudates may contribute to belowground associational effects, and should be tested for causality in mechanistic studies. In contrast, we observed few strong correlations between specific metabolites and bacterial genera. Thus, we found some support for our second hypothesis. Two highly abundant exudates in particular, malic acid and stearic acid, correlated with overall shifts in the focal plant's rhizosphere communities, suggesting they play a role in shaping communities. Both compounds are commonly reported root exudates (Aulakh et al., 2001; Liu et al., 2015), and malic acid has been identified as a chemoattractant for beneficial rhizosphere bacteria (Rudrappa et al., 2008; Ling et al., 2011; Jin et al., 2019).

We also cannot ignore several methodological factors that may have affected our ability to test the linkage between exudates and neighbor-induced shifts in bacterial communities. First, because root exudates are suggested to have the greatest influence on bacteria near root hairs (Rüger et al., 2021), our sampling of the entire root system may have weakened our ability to detect strong correlations. Second, microbial spillover from neighbor roots may have contributed to shifts in the rhizosphere community. Lastly, it is possible that microbial excretion or consumption of metabolites altered the exudate profiles. But thorough root washing, and the generally higher production of plant-derived metabolites versus microbially-derived per unit volume probably made this only a small factor for highly-abundant metabolites (Williams et al., 2021a). The abundance of malic acid, in particular, can increase with root damage (Williams et al., 2021a), potentially elevating its statistical impact on the microbial community.

When we added malic acid directly to soil in incubations, we also saw changes in microbial communities, but the bacterial genera enriched in the high malic acid incubations were not the same genera correlated with malic acid in the greenhouse experiment. The observed inconsistencies in bacterial enrichment with malic acid are likely driven by artifacts from distinct experimental conditions in the greenhouse and laboratory, including the use of different soil substrates in either experiment. These inconsistencies could also suggest that other factors beyond selection by malic acid are shaping neighbor-induced changes in microbial communities. For instance, we found an unexpected correlation between malic acid exudation and the abundance of the bacterial genera *Methylophilus.* This group consists of facultative methylotrophs that would not utilize malic acid as a primary energy source (Jenkins et al., 1987), and may indicate that other factors, such as shifts in soil nutrients, drove this correlation.

The soil incubation further highlighted that neighbor-induced changes in root exudates may have even more pronounced effects on nutrient cycling, than on bacterial assembly. We found that the greater addition of malic acid increased soil DOC and TN and decreased microbial biomass C:N. While these results may be influenced by differences in sugar content between the high- and low-malic acid treatments, which could alter microbial growth and N-use (Schneckenberger et al., 2008; Cao et al., 2021), our results are consistent with previous studies showing that organic acids stimulate greater release of microbially-available N than sugars (Yuan et al., 2018). Organic acids can stimulate the release of C and N from soil through two mechanisms: first, they can stimulate microbial enzyme production, which then releases mineral-bound nutrients, and second, they can directly liberate organic compounds from mineral soils, making them more available to microbes (Keiluweit et al., 2015; Jilling et al., 2021). These studies used oxalic acid, another commonly exuded organic acid, but we show that malic acid could play a similar role in nutrient mineralization in soils. In sum, the soil incubation suggests that neighbor-induced changes in exudates play an important role in nutrient cycling, and that the correlations between malic acid and bacterial community structure may be driven by the microbes' response to soil N, rather than a direct chemotactic response to the exudate.

NEIGHBOR EFFECTS ARE GREATEST DURING COMPETITION AND NUTRIENT-LIMITATION

In support of our third hypothesis, we found that neighbor-induced changes in bacterial communities were greatest during strong competition. All neighbors had a competitive effect on focal plant biomass (negative RII), but the forb *R. hirta* caused the greatest reduction in focal plant biomass and had the strongest effect on the rhizosphere community. Patterns in focal plant exudates were similar, but also responded to the less competitive *K. macrantha* neighbor. This suggests that while microbiomes are strongly influenced by characteristics of strong competition, such as nutrient stress and reduction in biomass, other mechanisms contributed to shifts in the exudates, such as aboveground signaling (Li et al., 2020; Kong et al., 2021) or neighbor detection (Biedrzycki and Bais, 2010; Kong et al., 2018). Still, we cannot, distinguish how the observed effects on rhizosphere structure are impacted by neighbor identity and competition. *R. hirta* was both the only forb neighbor and the largest neighbor, and caused the greatest reduction in focal plant biomass, tissue N content and soil moisture, and each mechanism may have contributed to its greater effect on focal plants.

Abiotic factors in particular can directly affect microbial community structure (Fierer, 2017; Naylor and Coleman-Derr, 2018) and may also drive indirect, host-mediated shifts in microbiomes by altering root exudates (Weidenhamer et al., 2019; Williams and de Vries, 2019; Smercina et al., 2020). We saw that focal plant C:N explained variation in both the bacterial community and root exudates, suggesting that N competition was particularly important to *R. hirta*'s strong effect on the focal plant. Surprisingly, soil nitrate levels did not differ by treatment, but the drier soil of the *R. hirta* treatment may have reduced focal plant N uptake (Gonzalez-Dugo et al., 2012; Bista et al., 2018). This N and water limitation likely increased exudation of compounds related to plant stress response. For instance, glycerol 3-phosphate (G3P), which was exuded more next to *R. hirta,* is shown to increase in both plants (Shen et al., 2006) and microbes (Albertyn et al., 1994) during osmotic stress. There may even be a link between G3P and the recruitment of beneficial, drought-tolerant microbes (Xu et al., 2018), as well as host immunity against pathogens (Chanda et al., 2011; Mandal et al., 2011). In this study we did not find strong correlations between G3P and bacterial taxa, suggesting that more studies are needed to elucidate the role that G3P exudation plays under stress and, specifically, if it influences microbial assembly.

P. virgatum also exuded more organic acids (fumaric acid, malic acid, and saccharic acid) while neighboring *R. hirta*, likely due to nutrient limitation. Plants release more organic acids under a variety of nutrient stresses (K+, P, N, Ca^{2+}, Zn^{2+}) (Jones, 1998; Panchal et al., 2021), so though we know that N was limited, other nutrients may have also triggered this response. Several recent studies show that our focal plant, *P. virgatum,* exudes more organic acids and fewer carbohydrates in N-limited, sterile, conditions (Smercina et al., 2020), and that these organic acids increase soil DON and N-mineralization, but not biological N-fixation (Liu et al., 2022). Accompanied with our soil incubation results, this shows that organic acids may do more to alleviate plant N stress through physical liberation of minerally-bound N, rather than through recruitment of beneficial microbes, such as free-living N-fixers.

Finally, in addition to *R. hirta's* distinct effect on soil resources, it was also the largest neighbor plant, which may have contributed to its strong effects on the focal plant's rhizosphere community. With a root system that was ten times larger than the other neighbors, *R. hirta's* roots likely had greater overlap and microbial spillover with the focal plant rhizosphere. In fact, the bacterial genera that were more prevalent in the focal plant's rhizosphere near *R. hirta* were also most abundant in the *R. hirta* monoculture (Figure 2.S4), suggesting a role of microbial spillover. Higher exudation rates in grassland forbs than grasses (Williams et al., 2021b) may have also strengthened its effect on the focal plant's rhizosphere. *R. hirta's* effect on the focal plant's non-dominant taxa, however, was not driven by microbial spillover, as the non-dominant taxa represented a novel community distinct from either the *R. hirta* or *P. virgatum* monoculture. This result indicates that mechanisms other than microbial spillover, perhaps shifts in neighbor signaling, drive changes in non-dominant rhizosphere taxa. Lastly, these subtle, but clear shifts in non-dominant taxa may also help explain why neighborhood effects are seldom noticed, yet why some plant pairings have non-additive effects on microbial functions, such as nutrient cycling (Betencourt et al., 2012; Li et al., 2016; Sekaran et al., 2020).

CONCLUSION

In summary, we show that neighbor plants influence one another's rhizosphere assembly, especially during strong neighbor competition. This suggests that studies on isolated plants may not be predictive of rhizosphere assembly in natural conditions. We found evidence for multiple mechanisms contributing to neighbor-induced changes in the rhizosphere bacterial communities. While the exudate profile was strongly correlated to the overall microbiome, suggesting that exudates may play a role, we could not repeat the same taxonomic shifts by manipulating a dominant exudate, and did not identify a causal link between these factors. In fact, the dominant exudate also increased soil N, suggesting that neighbor-induced changes in exudates may have even stronger effects on soil nutrients than microbial assembly. Still, future studies should explore the spatial and temporal scales at which neighbors affect exudates and rhizosphere taxa, as this likely influenced our ability to correlate shifts in taxa and exudates. Overlapping roots and microbial spillover also contributed to the strong neighborhood effects. Future studies with root barriers will help elucidate the relative role of microbial spillover and exudate-mediated microbial assembly on associational effects. Overall, this study highlights that exploring plant-microbial dynamics in mixed-species neighborhoods can help increase our understanding of the mechanisms that drive rhizosphere assembly in nature, as well as improve our ability predict and manage for beneficial microbial interactions.

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ADDITIONAL INFORMATION

Code and Data are openly available: code and analyzed data (https://github.com/TaylerUlbrich/NeighID_Switchgrass); Raw bacterial fastq files (NCBI accession number PRJNA773254).

CHAPTER THREE: NEIGHBORHOOD EFFECTS, AND THE FACTORS MEDIATING THEM, DIFFER FOR RHIZOSPHERE AND ROOT MICROBIOME ASSEMBLY

ABSTRACT

Root and soil microbes provide many beneficial functions for plant hosts and being able to capitalize on these benefits demands an understanding of microbiome assembly. Studies show beyond the host plant, neighbor plants can also impact one another's microbiome assembly, but the patterns and mechanisms vary, and controlled studies are needed to elucidate the relative role of the host- and neighbor-plant. To this end, we used a controlled rhizobox experiment that manipulated the neighbors of a focal plant, *Panicum virgatum.* We used root barriers that reduced the 'spillover' of novel neighbor microbes, as well as fine-scale sampling of root exudates and microbiomes to isolate the relative role of the host- and neighbor-plant in microbiome assembly within and on the root, as well as in the soil near the root, 'rhizosphere soil'. We found that neighbors impacted the focal plant root-associated microbiomes, but not the rhizosphere soil microbiomes or root exudates. This pattern was true for fungi and bacteria. The neighbor-induced shifts in the focal plant root-associated microbiomes occurred even in the absence of overlapping roots, suggesting that the host plant plays a strong role in mediating neighbor effects on root microbiome assembly. In contrast, the rhizosphere soil microbiomes were not affected by neighbor, likely because there were no neighbor effects on the focal root exudates, and the neighbors also did not assemble distinct microbiomes, so could not introduce novel taxa during root overlap. Overall, this study shows that the neighborhood effects on root-associated and rhizosphere soil microbiomes differ, and while the host plant plays a role in mediating root assembly, neighbor-effects on rhizosphere communities depend on the introduction of novel taxa from species-specific rhizospheres.

INTRODUCTION

Plant hosts associate with soil and root microbiomes that influence their growth and resistance to stress (Berendsen et al., 2012; de Vries et al., 2020). Capitalizing on microbiome benefits to enhance plant growth and resilience demands an understanding of microbiome assembly (Quiza et al., 2015; Munoz-Ucros et al., 2021). It is broadly accepted that microbiomes are influenced by soil conditions as well as host-specific factors (Berg and Smalla, 2009). Root-derived metabolites play a primary role in filtering microbes in a gradient of decreasing diversity and increasing plant-association, from the surrounding bulk soil to soil near the plant root, the "rhizosphere", and finally inside the root, the "endosphere" (Bulgarelli et al., 2013; Reinhold-Hurek et al., 2015). Beyond the host plant, it is also possible that microbiome assembly is influenced by neighboring plants. Indeed, neighborhood effects, defined as the influence of neighbors on a focal plant's fitness or interactions with other organisms, have been extensively studied for pathogens and herbivores (Barbosa et al., 2009; Underwood et al., 2014) and, more recently, have also been shown to affect belowground microbiome assembly. This can occur in many settings, including in native plant communities (Bakker et al., 2013; Morris et al., 2013; Horn et al., 2017; Vieira et al., 2019), during plant invasions (Mummey et al., 2005; Hawkes et al., 2006; Broz et al., 2007), and in agricultural landscapes (Song et al., 2007; Zhang et al., 2010; Gelfand et al., 2011; Li et al., 2016). However, controlled studies are needed to decipher the dominant mechanisms that underlie this pattern (Li et al., 2016; Chen et al., 2020; Kong et al., 2021).

Patterns of neighbor-induced changes in microbiome assembly vary widely, suggesting that there are several mechanisms at play. In some cases, a neighbor plant that is more competitive for nutrients can alter local abiotic conditions, such as soil nutrients or water availability, which then impacts the focal plant microbiome (Hortal et al., 2017a; Ulbrich et al., 2022). Changes in abiotic conditions can have direct effects on microbial communities (Fierer et al., 2007; Naylor, 2017), but can also drive host-mediated shifts in microbiomes, such as when abiotic conditions alter root exudates or plant immune responses (Fitzpatrick et al., 2020). Other times, neighborhood effects may be the result of "microbial spillover", or the introduction of novel neighbor microbes into a focal plant rhizosphere. This could occur through several mechanisms, such as dispersal through fungal hyphae that extend between neighbor roots (Yang and van Elsas, 2018), or when neighbor roots overlap and, therefore, are more likely to share rhizosphere microbes (Hausmann and Hawkes, 2009; Ulbrich et al., 2022). Spillover may result in the focal plant's rhizosphere microbiome resembling that of its neighbor (Hausmann and Hawkes, 2009; Hortal et al., 2017a; Ulbrich et al., 2022). Finally, it is also possible that signals involved in species-specific plant recognition drive changes in the plants' microbial communities. For instance, cyanide production from a neighboring Cassava plant (*Manihot esculenta*) has been shown to stimulate ethylene production from neighbor peanut plants (*Arachis hypogaea*) with subsequent shifts in the peanut rhizosphere soil microbiome (Chen et al., 2020). Altogether, these studies show that plant neighbors affect host microbiome assembly through many mechanisms, but the relative impact of the host plant versus external factors (e.g., spillover, abiotic conditions) on neighborhood effects needs to be elucidated.

Neighborhood effects have been observed in plant roots and rhizosphere soils, as well as for fungi and bacteria, but it is plausible that the mechanisms driving neighbor-induced changes in these groups may differ. For instance, mycorrhizal fungi are a common group of fungi that respond to shifts in plant neighborhoods (Mummey et al., 2005; Hausmann and Hawkes, 2009; Horn et al., 2017), possibly because their hyphal networks can connect neighboring root systems, exchanging nutrients and signals between neighboring plants (He et al., 2003; Babikova et al., 2013). Therefore, it is possible that neighbor plant mycorrhizae may alter a focal plant's microbiome even at a distance, via hyphae. Hyphal networks have also been shown to facilitate the migration of more dispersal limited soil microbes, like bacteria and yeasts (Yang and van Elsas, 2018). In these cases, bacteria and yeasts could also be introduced to a focal plant's microbiome via hyphae, but because the likelihood of hyphal dispersal varies among species and soil moisture conditions (Kohlmeier et al., 2005; Worrich et al., 2016), one may expect microbial spillover via root overlap to have a stronger effect on assembly.

There are also known differences in the assembly of root and rhizosphere soil microbiomes that may be reflected in neighborhood effects. Rhizosphere soil microbiome composition is controlled by the initial composition of the bulk soil community (Vieira et al., 2019), root exudate composition (Badri et al., 2012; Lebeis et al., 2015; Zhalnina et al., 2018), by edaphic factors (Nuccio et al., 2016; Ulbrich et al., 2021), and via complex cross-kindom biochemical dialogues (Venturi and Keel, 2016). In contrast, endophytes, or microbes that live within the root, may originate from the seed (Truyens et al., 2015) and are also filtered from rhizosphere soil through species-specific signaling and immune system responses (Reinhold-Hurek and Hurek, 2011; Edwards et al., 2015b). Therefore, while spillover from neighbor plants or shifts in abiotic conditions can affect the recruitment pool for endophytes (neighbor-mediated mechanisms), the host plant is likely the ultimate regulator of which microbes colonize root interiors. For these reasons, we predict that neighborhood effects on root microbes will be mediated mostly by the host plant, for instance through changes in host physiology or signaling, while the assembly of microbes in rhizosphere soil will be influenced by both host- and neighbor-mediated mechanisms. In fact, in our previous study, we found that neighborhood effects on rhizosphere bacteria were mediated by changes in root exudates (host-mediated) and soil moisture (neighbor-mediated), as well as spillover of neighboring soil microbes (neighbor-mediated) (Ulbrich et al. 2022).

To determine the relative role of host- and neighbor-mediated mechanisms in neighborhood effects, studies need to address several challenges. A first challenge is the need to identify if novel taxa are introduced to a focal plant rhizosphere primarily through microbial spillover, or through host-mediated avenues, such as via recruitment with changes in root exudates. A second challenge is with the scale in which most studies sample microbial communities and root exudates. Often, microbiome samples and root exudates are collected from the entire root system, but this pooling approach homogenizes potentially meaningful spatial variation in microbiomes (Munoz-Ucros et al., 2021) and root exudates (Canarini et al., 2019). For instance, nitrate additions to one side of a root system can stimulate localized increases in exudation rates and bacterial colonization (Paterson et al., 2006). It is plausible that similarly localized shifts in microbiome assembly could occur in mixed plant neighborhoods, but that they would only be detected with spatially-explicit, fine-scale sampling efforts.

Here, we attempted to overcome challenges to understanding the mechanisms driving neighborhood effects on rhizosphere microbiomes. We manipulated a focal plant's neighbor and used root barriers to reduce the effect of microbial spillover (primarily reducing spillover from root overlap), as well as fine-scale sampling of root exudates and microbiomes in two rhizosphere compartments to look for localized neighborhood effects. We assess the microbiomes within the rhizosphere soil compartment, defined as soil that was adhering to or within 2 mm of a root, as well as the microbiomes of the rhizoplane and endosphere compartments together, which we call the root-associated microbiome. Finally, unlike most greenhouse studies on neighborhood effects that inoculate potting media or sand with a small percent of field soil (Hausmann and Hawkes, 2009; Cavalieri et al., 2020; Mony et al., 2021; Ulbrich et al., 2022), we used 100% field soil to ensure that outcomes were more representative of what may occur in natural settings.

Using this design, we asked three main questions: 1) To what extent is rhizosphere microbiome assembly (soil and root-associated) distinct among host species?, 2) Do neighbor interactions alter a focal plant's microbiome assembly? And, 3) what is the relative role of the host plant versus microbial spillover in mediating neighbor-induced changes in microbial assembly? First, we hypothesized that both rhizosphere compartments would have distinct microbiomes among plant species, but that the species-effects would be stronger for the root-associated communities. Second, we hypothesized that neighbor identity would alter the composition of a focal plant's microbiomes, but that the mechanisms driving these shifts would differ by plant compartment. Specifically, we hypothesized that neighbor-induced shifts in the root-associated compartment would be driven primarily by the host plant, such that the effect would be present even in the absence of microbial spillover during active root interactions (barrier between plants). In contrast, we predicted that neighbor-induced shifts in the rhizosphere compartment would be most strongly influenced by microbial spillover during active root interactions, and that neighbor-induced changes in abiotic conditions and focal plant root exudates would also play significant, though more minor roles. If this is true, and microbial spillover is the prominent mechanism mediating neighborhood effects in the assembly of rhizosphere soil microbiomes, then we would expect to see a stronger neighborhood effect when there are active root interactions (no barrier between plants), compared to when there are not.

METHODS

STUDY SPECIES

The focal species used in this study was switchgrass (*Panicum virgatum* L. var. Cave-in-rock), which is a C4 perennial grass native to tallgrass prairies and is also a candidate bioenergy crop (McLaughlin and Kszos, 2005). Recent studies suggest that *P. virgatum's* root exudates and rhizosphere microbiomes vary depending on the identity of its neighbor (Ulbrich et al., 2022). Neighbor plants can also alter a focal switchgrass plant's belowground carbon inputs, perhaps because neighbor identity impacts the potential for interplant carbon transfer (Kravchenko et al., 2021). In this study, we seek to isolate the mechanisms mediating previously-observed neighbor-effects on *P. virgatum's* rhizosphere microbiome. The neighbor species used in this study were conspecific *P. virgatum* (hereafter, PV), as well as two perennial prairie species known to co-occur with PV: *Rudbeckia hirta* L. (RH, a forb) and *Lespedeza capitata* Michx (LC, a legume).

RHIZOBOX DESIGN & SOIL PROPERTIES

Rhizoboxes were designed to manipulate a focal plant's neighbor context (described below in 'Plant Neighbor Treatments'), as well as the capacity for the neighboring roots to interact (described below in 'Barrier Treatment'). The boxes had three sections with a focal plant in the center and neighbors on either side (Figure 3.1). The boxes (54 cm wide x 30 cm tall x 4 cm deep) were made of acrylic and polycarbonate and the transparent sides were placed at 60° angle to encourage root growth towards the front, facilitating easier sampling (Supplementary Figure 3.1 for design; See Supplemental Methods for additional details).

Soil for the experiment was collected from the Great Lakes Bioenergy Research Center Marginal Land Experiment site in Escanaba, Michigan, USA (45°45'49"N, 87°11'14"W). These soils are characterized as a sandy loam (Inceptic Hapludalf Alifsol) with approximately 1.73% organic carbon (Kasmerchak and Schaetzl, 2018). Wheat was growing at the time of soil collection. Soil was sampled to a depth of 20 cm. The soil was then air dried and sieved (2 mm) before filling into rhizoboxes at a consistent bulk soil density and soil moisture (1.28 g cm^{-3} and 20% volumetric water content).
After filling the rhizoboxes, one week old seedlings were transplanted into each of the three sections. Plants were replaced for up to two weeks, if mortality occurred, and grown for 14 weeks in their neighbor treatments. The rhizoboxes were positioned in the greenhouse in a complete randomized blocked design to control for heterogeneity in greenhouse, with temperatures controlled at a maximum of 29 °C during the day and minimum of 20 °C at night with 16 hours of artificial lighting. They were

watered with RO water as needed and fertilized twice during the experiment to adjust for potassium deficiency in the soil (KH_2PO_4 to the equivalent of 56.04 Kg P Ha^{-1}).

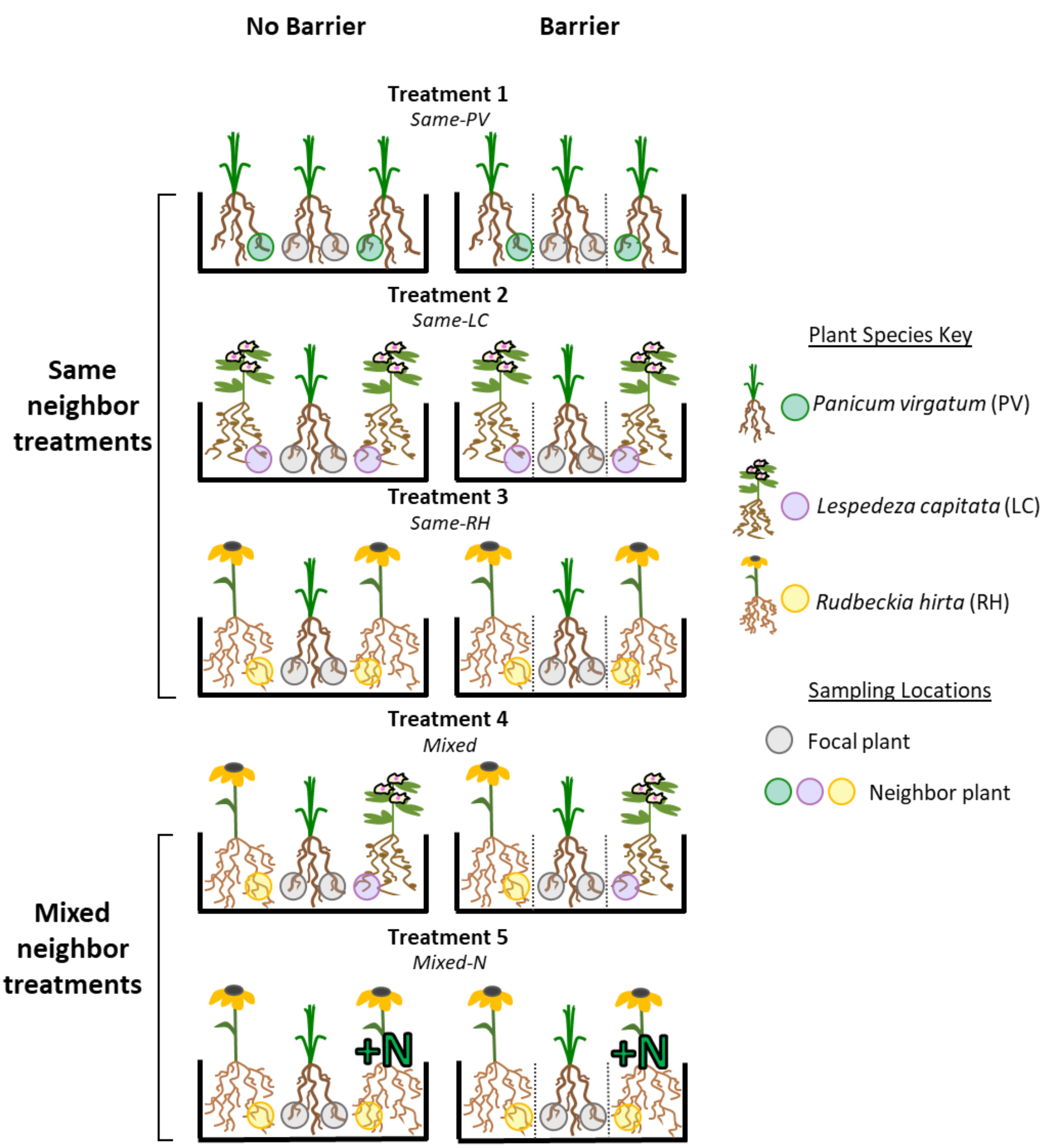

Figure 3.1. Experimental design showing five plant treatments that manipulated neighbor identity surrounding a focal plant (*Panicum virgatum*) and two barrier treatments that either allowed active root interactions (No Barrier) or prevented active root interactions (Barrier; 35 µM mesh barrier). Circles represent sampling locations, color coded by neighbor identity (green = *Panicum virgatum*, purple = *Lespedeza capitata*, yellow = *Rudbeckia hirta*) and gray circles in the center represent two samples taken from either side of the focal plant. Treatment numbers, labels, and colors are consistent in other figures (light gray = focal plant, filled in colors = neighbors).

BARRIER TREATMENT

Root interactions between the focal plant and neighboring species were manipulated with two barrier treatments. The "No Barrier" treatment had no mesh barrier and allowed active root and mycorrhizal interactions between the plants (Figure 3.1, first column). The "Barrier" treatment prevented active root interactions with a 2 cm soil gap and mesh barrier (35 µM, ELKO Filtering Co.), but still allowed mycorrhizal hyphal exchanges (hyphae 2-20 µM; Friese and Allen, 1991) (Figure 3.1, second column). Within each barrier treatment, there were five neighbor treatments, described below. See Supplementary Figure 3.1 for detailed picture of barriers.

PLANT NEIGHBOR TREATMENTS

The focal plant, PV, was surrounded by five different neighbor combinations. Three treatments, hereafter referred to as "same neighbor treatments", included two plants of the same species – *P. virgatum* (PV), *R. hirta* (RH), or *L. capitata* (LC) – on either side of the focal plant (Figure 3.1, first three rows). The other two treatments, hereafter referred to as "mixed-neighbor treatments" varied the neighbor conditions on either side of the focal plant (Figure 3.1, last two rows). In the first mixed-neighbor treatment, the focal was neighbored by RH and LC; this allowed us to determine if neighbor identity has neighbor-specific, localized effects on the focal plant. The second mixed-neighbor treatment (treatment 5) included RH as a neighbor on either side, but one of the neighbors was enriched with nitrogen (N) in their tissues (RH+N). For two weeks, N (100 ppm N –NH_4NO_3) was added to the RH+N plant through scintillation vials attached to their leaves, following methods of (Rasmussen et al., 2019), and then for three weeks after, the plant received the same N solution directly to the crown of the plant weekly. This allowed us to evaluate how neighbor N status, separate from neighbor identity, influences neighborhood effects. Each treatment was replicated five times in either barrier treatment, with a total of 50 rhizoboxes (5 treatments x 2 barriers x 5 replicates = 50 total, Figure 3.1).

To address questions pertaining to a separate part of this study, the focal plants were subject to ^{15}N-NH_4NO_3 additions to the leaves (Rasmussen et al., 2019), as well as ^{13}C-CO_2 pulses (8000 ppm CO_2, three 6-hour pulses total, occurring every 7 days for 3 weeks) following methods of (Kravchenko et al., 2021) . These conditions may have played a role in neighbor interactions and microbiome assembly, though most previous literature highlights the effect of elevated CO_2 on root exudates and microbiome assembly over extended periods (months), not single pulse events (Drigo et al., 2008; Phillips et al., 2009).

PLANT HARVEST AND SOIL ANALYSES

The rhizoboxes were harvested when the plants were 14 weeks old. The harvest took five days and the boxes were sampled according to their experimental blocks. First, the rhizosphere soil (see Microbiome Collection below) and root exudates were collected (see Exudation Collection below), followed by root segments for root-associated microbiome analyses. Prior to the root exudate collection, soil samples were collected from every sampling point in the rhizobox (see shaded circles in

Figure 3.1) and used for gravimetric soil moisture analyses. Each plant's aboveground and belowground biomass was collected, dried (60 °C temp for 3 days) and weighed; a subsample of the tissue was used for carbon and nitrogen analyses (Costech Analytical ECS 4010 CHNSO Analyzer).

MICROBIOME COLLECTION

Rhizosphere soil and root-associated microbiome samples were collected from two intact roots from either side of the focal plant, as well as a single root from either neighbor plant (see sampling points, Figure 3.1). The roots were carefully excavated and the rhizosphere soil within approximately 2 mm of the roots was collected with sterile brushes, flash frozen in liquid nitrogen, and stored at -80 °C for DNA extractions. The flash-frozen rhizosphere soil samples were freeze-dried (Harvest Right Scientific Freeze Dryer; following protocol (Weißbecker et al., 2017) and stored at 4 °C prior to DNA extractions, described below. From the focal plant, the same four roots used for rhizosphere soil collection (two focal left and 2 focal right) were also used for collecting root exudates. This sampling creates a tightly coupled, paired dataset for the rhizosphere soil microbiome and root exudate data. We also collected roots for root-associated microbiome analyses, which were not surface-sterilized and so represent the plant endosphere and rhizoplane communities. Following root exudate collection (detailed below), a root segment with visible root hairs (< 2 mm in diameter and approximately 2.5 inches long) was clipped from similar sampling locations as the rhizosphere soil samples, though not always from the same root as the rhizosphere soil samples and root exudates. The roots were rinsed with a 0.05% Tween 20 wash to remove soil debris and then rinsed with sterile water, flash-frozen with liquid N, and then vacuum dried (speedvac, Thermo Fisher) for four hours before grinding with TissueLyser II (Qiagen). Despite attempts to standardize root size, there was still some variation in root segment sizes, especially among plant species, which may have influenced downstream sequencing. We controlled for this sample variation by rarefying the microbial dataset, as described below.

ROOT EXUDATE COLLECTION

Soluble root exudates were collected using a modified *in situ* soil-hydroponic method from (Phillips et al., 2008). We collected root exudates from only the focal plant in all treatments. Two intact roots from either side of the focal plant (gray sampling points, Figure 3.1) were carefully excavated from the soil, removed of adhering soil for rhizosphere soil samples (described previously), rinsed with a weak salt buffer solution (10 ml 10 mM KH_2PO_4, 10 ml 20 mM K_2SO_4, 10 ml 30 mM $CaCl_2$), and 'incubated' for four hours in 15 mL tubes (syringes with the plunger removed) with the same buffer solution and acid washed glass beads (0.5-2 mm). It is possible that the roots experienced minor damage during excavating and rhizosphere soil collection, but likely far less than occurs in other common soil-hydroponic collection methods (Williams et al., 2021a). The four-hour root exudate collection period is recommended to limit microbial degradation and consumption of metabolites in non-sterile systems (Oburger and Jones, 2018). Throughout the exudate collection, the open side of the rhizobox was covered with damp paper towels to limit light exposure, as well as prevent the roots from drying out. At the end of the incubation, the roots were cut from the plant, and the exudates were flushed with an additional 20 mL of the salt solution. Exudates from the two roots on either side of the focal plant were combined (n = 2 exudate samples per focal plant), filtered (0.45 µM and 0.22 µM PVDF filters), separated into two replicates (10 mL used for LCMS analysis), flash-frozen in liquid N, and subsequently lyophilized (Harvest Right Scientific Freeze Dryer; following protocol Weißbecker et al., 2017). Each day we also collected a methodological blank, which involved the same incubation process but without a root, thus allowing us to account for any metabolite contamination from the tubes, filters, and collection process. Each root segment used for exudate collection was stored in 50% EtOH and then weighed to standardize the root exudate data, described below.

ROOT EXUDATE PREPARATION, LC-MS, AND NORMALIZATION

The freeze-dried root exudates were prepared for untargeted analysis with liquid chromatography mass spectrometry (LC-MS) by resuspending them in 80% methanol, drying with a speedvac (Thermo Savant DNA 120), and reconstituting them in 50% methanol containing 100 nM telmisartan (internal standard). The samples (5 µL) were processed using a Waters Acquity UPLC interfaced with a Waters Xevo G2-XS QTof mass spectrometer and a Waters Acquity UPLC HSS-T3 column (2.1x100 mm). Compounds were ionized with electrospray ionization operating in either positive or negative ion mode. Peak alignment and picking were performed using Progenesis QI software (Nonlinear Dynamics, Waters) with a pooled sample used as the alignment reference. Unique metabolite features identified from the positive and negative LC-MS modes were merged into a single database. Though this may result in some redundancy in features, it provides a more complete representation of the plants' exudate profile. Additional details about LC-MS protocol and peak-picking are described in the supplemental methods.

Statistical analysis (described below) revealed that the composition of the root exudates varied across the five sampling days throughout the harvest (PERMANOVA sampling day as main effect, F = 7.91, $p < 0.001$, $R^2 = 0.26$, Figure 3.S3). Though the buffer was stored in a sterilized, plastic carboy and efforts were taken to keep the solution sterile, it is plausible that it became contaminated throughout the week and, therefore, that microbial metabolites were captured in our analyses, contributing to the sampling day effect. We tried to control for this by removing any metabolite features that were three times more abundant in the blanks than in the samples (maximum intensity in samples/maximum intensity in blank ≥ 3) (Weinhold et al., 2022) or only present in less than 10% of the samples. This resulted in a total of 2,040 metabolic features. The peak intensity of these features was standardized by the dry weight of the root segments used for the exudate collection and then log10 transformed to meet normality requirements with Euclidean-based statistics.

DNA EXTRACTION, ILLUMINA SEQUENCING, AND BIOINFORMATICS

DNA was extracted from ~ 0.1 g of the freeze-dried rhizosphere soil using the MagAttract PowerSoil Pro DNA Kit (Qiagen) and from the freeze-dried root samples with the Omega Mag-Bind Plant DNA Plus kit (Omega Bio-Tek). The KingFischer Flex purification system platform (Thermo Fisher) was used with both extraction kits. Each 96-well plate included negative-control blank extractions to remove contaminants, as described below (Davis et al., 2018). We characterized the bacterial communities by targeting the bacterial V4 region of the 16S rRNA gene (primers 515f/806r) (Caporaso et al., 2011) and the fungal communities by targeting the ITS2 rRNA region (Taylor et al., 2016). Bacterial and fungal multiplexed libraries were prepared with a three-step PCR protocol as previously described (Lundberg et al., 2013b; da Costa et al., 2022) and then sequenced on an MiSeq Illumina analyzer (v3 600 cycle kit, Illumina, USA) by the Research Technology Support Facility Genomics Core at Michigan State University (East Lansing, Michigan, USA). See Supplemental Methods for additional details the protocol used for library preparation.

The raw sequences were demultiplexed with default setting of Illumina Bcl2Fastq. The forward and reverse reads were merged for the rhizosphere soil bacterial dataset, as well as thefungal datasets (root-associated and rhizosphere soil). However, due to low quality reverse reads for the root-associated bacterial samples, we only used the forward reads for a combined rhizosphere soil and root-associated bacterial dataset (described in more detail below). Quality filtering (maxEE = 5, truncQ = 2), chimera removal, and taxonomic assignment using silva 138 for bacteria and UNITE 8.2 for fungi was conducted with the R package “dada2” (Quast et al., 2012; Callahan et al., 2016; Nilsson et al., 2018). The ASVs were clustered into operational taxonomic units (OTUs) with 99% similarity with packages DECIPHER and Biostrings in R (Wright, 2016; Pagès et al., 2021).

The library preparation and sequencing runs were separate for the root-associated and rhizosphere soil microbiomes, so sequences were filtered individually before merging into a combined dataset. For each, we removed contaminants (R package decomtam, method = “prevalence”, threshold = 0.1), rare OTUs (present in < 5 samples), low-coverage samples and, for bacteria, non-bacterial reads (archaea, chloroplast, mitochondria). Rarefaction curves obtained from the bacterial and fungal datasets approached saturation, indicating that sequencing depths were sufficient (Supplementary Figure 3.S2A, B). Still, to account for read depth differences among the neighbor treatments and rhizosphere compartments (rhizosphere soil and root-associated), we rarefied the datasets using the "rarefy_even_depth" function in the Vegan R package (Oksanen et al., 2018). The combined fungal dataset (root and soil) was rarefied to 22,547 reads. The root-associated bacterial dataset (forward reads only) had lower read depth due to loss of reads from chloroplasts and mitochondria. To avoid losing too many samples, while still accounting for differences in reads between the two rhizosphere compartments, , the combined bacterial dataset (root-associated and rhizosphere soil)) was rarefied to 1,444 reads (lost 22 root-associated and 1 rhizosphere soil samples), which likely reduced the diversity of the bacterial microbiomes(Supplementary Figure 3.2C). The rhizosphere soil bacterial samples did have adequate read coverage, so we rarefied this separate dataset to 4,814 reads. The phyloseq (McMurdie and Holmes, 2013) and dplyr (Wickham et al., 2022) R packages were used for data filtering and organization. Additional details about microbiome filtering provided in Supplemental methods "*Microbiome Bioinformatics and Filtering*".

STATISTICAL ANALYSES

First, we analyzed data collected from the neighbor plants to test whether different plant species varied in size, nutritional status (shoot CN), or if they assembled distinct microbiomes (species-effects). Second, we analyzed the focal plant (PV) within each neighbor treatment to evaluate neighbor-effects on the focal plant biomass, nutritional status, root exudates, and microbiomes. For Mixed treatments, in which samples were collected from either side of the focal plant and a different neighbor (microbiome and root exudate data, gravimetric water content and root CN), we evaluated the effect of either side of the Mixed treatment using a combined term for plant treatment and neighbor identity (e.g. Treatment 4 Mixed becomes Mixed,RH or Mixed,LC). In contrast, in the Same treatments, because the focal plant was exposed to the same neighbors on either side, both focal plant samples were considered replicates of a single treatment.

For univariate data (plant and soil data, alpha diversity metrics), comparisons among plant treatments and barriers were assessed using a mixed-model analysis of variance (ANOVA) that included plant treatment, barrier, and their interaction as fixed effects, and block as a random, nested factor. Shannon diversity models also included read depth as a covariate, and were analyzed on unfiltered (no removal of low-read OTUs) and unrarefied datasets because removal of sparse OTUs is problematic for alpha-diversity metrics (Willis, 2019; Kleine Bardenhorst et al., 2022). Root exudate alpha diversity metrics were calculated with sampling day, rather than replicate as a random factor. The lme4 and emmeans R packages were used for the univariate mixed-model fitting, ANOVA (function emmeans joint_tests) and post-hoc pairwise comparisons (Benjamini-Hochberg False Discovery Rate, FDR, α = 0.05)(Bates et al., 2015; Lenth, 2019). If the data did not meet model assumptions, it was transformed (log or square root) and, in some cases, factors were weighted for unequal variance using the "weights" function in the R package lme4 (Bates et al., 2015).

All multivariate analyses of the microbiomes were performed on Bray-Curtis dissimilarity matrices from the rarefied datasets, and all multivariate analyses of the exudate data were performed on the log10-transfomed, Euclidean distance matrices. Plant and Barrier treatment effects were evaluated using permutational multivariate ANOVA tests (PERMANOVA, n = 999) and permutational multivariate Levene's test for homogeneity of variances (PERMDISP, n = 999), followed by post-hoc pairwise comparisons with Monte-Carlo adjustment with Primer (version 6 with PERMANOVA +) (Anderson et al., 2008). The models included plant treatment, barrier, and their interaction as fixed effects, and experimental replicate as a random, nested factor. For the root exudates, because the effect of sampling day was greater than that of replicate (PERMANOVA, replicate as a main effect, F = 3.184, $p < 0.001$, $R^2 = 0.13$), we used sampling day rather than replicate as the random factor. To further investigate the degree of species-effects within root and rhizosphere microbiomes (Question 1), as well as if there are localized neighborhood effects within the focal plant (Question 2), we calculated the Bray-Curtis dissimilarity between all sampling points within a sampling box (see shaded circles in Figure 3.1). These comparisons were then categorized as Intra-Plant (two samples collected within the same focal plant), Intra-Species (two samples collected from different plants of the same species), or Inter-Species (two samples collected from different species). We also compared the two rhizosphere compartments by calculating the percent of reads and OTUs that were present in both the root-associated and the rhizosphere soil microbiomes within a single sampling point.

Finally, to investigate the mechanisms contributing to neighborhood effects (Question 3), we explored how the focal plant microbiomes were impacted by microbial spillover during active root overlap, abiotic soil properties, and root exudates. The potential for microbial spillover was calculated by comparing the proportion of focal plant OTUs and reads that were shared with its neighbor on either side in the same rhizobox. If spillover occurred, and was mediated by root overlap, we would expect to see a higher percentage of shared reads and OTUs when there were active root interactions (No Barrier). Second, we used distance-based redundancy analysis (dbRDA) to determine how plant and soil characteristics – including focal plant aboveground and belowground biomass, focal root and shoot CN, and GWC – contributed to variation in the focal plant microbiomes. All variables were centered, scaled, and forward-step selection was used to identify the most parsimonious models, and replicate was included as a conditional factor. A similar approach, using redundancy analysis (RDA, Euclidean Distance) with sampling day as a conditional factor, was used to evaluate what soil and plant properties influenced the focal plant root exudates. Lastly, to evaluate the relationship between focal plant microbiomes and root exudates we looked for correlations between ordinations of the microbiomes (Bray-Curtis, PCoA) and root exudates (Euclidean Distance, PCA) using the function "Protest" in the Vegan R package (Oksanen et al., 2018). All figures were made with the R package ggplot2 (Wickham, 2016).

RESULTS

RH IS THE LARGEST NEIGHBOR AND DECREASES FOCAL PLANT SOIL MOISTURE, BUT NOT BIOMASS

Both neighbor and barrier affected the focal plant's soil moisture (gravimetric water content; Figure 3.2A), but neither impacted focal plant biomass (Figure 3.2B,C). Focal plant soil moisture was lower across all treatments when there was no barrier. When neighbored by two RH plants (Same-RH and Mixed-N) the focal plant had, on average, 26% lower soil moisture than when neighbored by the other neighbor species (Figure 3.2A). Neighbor did not affect focal root or shoot CN (Supplementary Figure 3.5C,D; neighbor effect focal shoot CN F = 0.720, p = 0.586; focal root CN F = 1.25, p = 0.318). There were also no localized differences in focal plant CN or soil moisture in response to different neighbors in the Mixed treatments (pairwise p between either side of the focal plant in Mixed-N treatment 5: GWC p = 0.841, root CN p = 0.993; Mixed treatment 4: GWC p = 0.994, Root CN = 0.965).

Among the neighboring plants , the RH neighbor had the lowest soil moisture (Figure 3.2D), as well as the largest aboveground and belowground biomass (Figure 3.2E,F). Visually, RH was also the only neighbor to have consistent root growth into the focal plant section in the no barrier treatment, while this occurred only seldomly with PV neighbors and rarely with LC neighbors. RH neighbors had lower shoot CN, but root CN did not differ among the neighbor species (Supplementary Figure 3.4A,B; species effect shoot CN F = 16.91, p < 0.0001; species effect root CN F = 1.90, p = 0.122).Even within the Mixed-N treatment (treatment 5), where N was added to one RH plant, we did not observe any significant differences in tissue CN or biomass (Figure 3.2E,F; Supplementary Figure 3.5A,B).

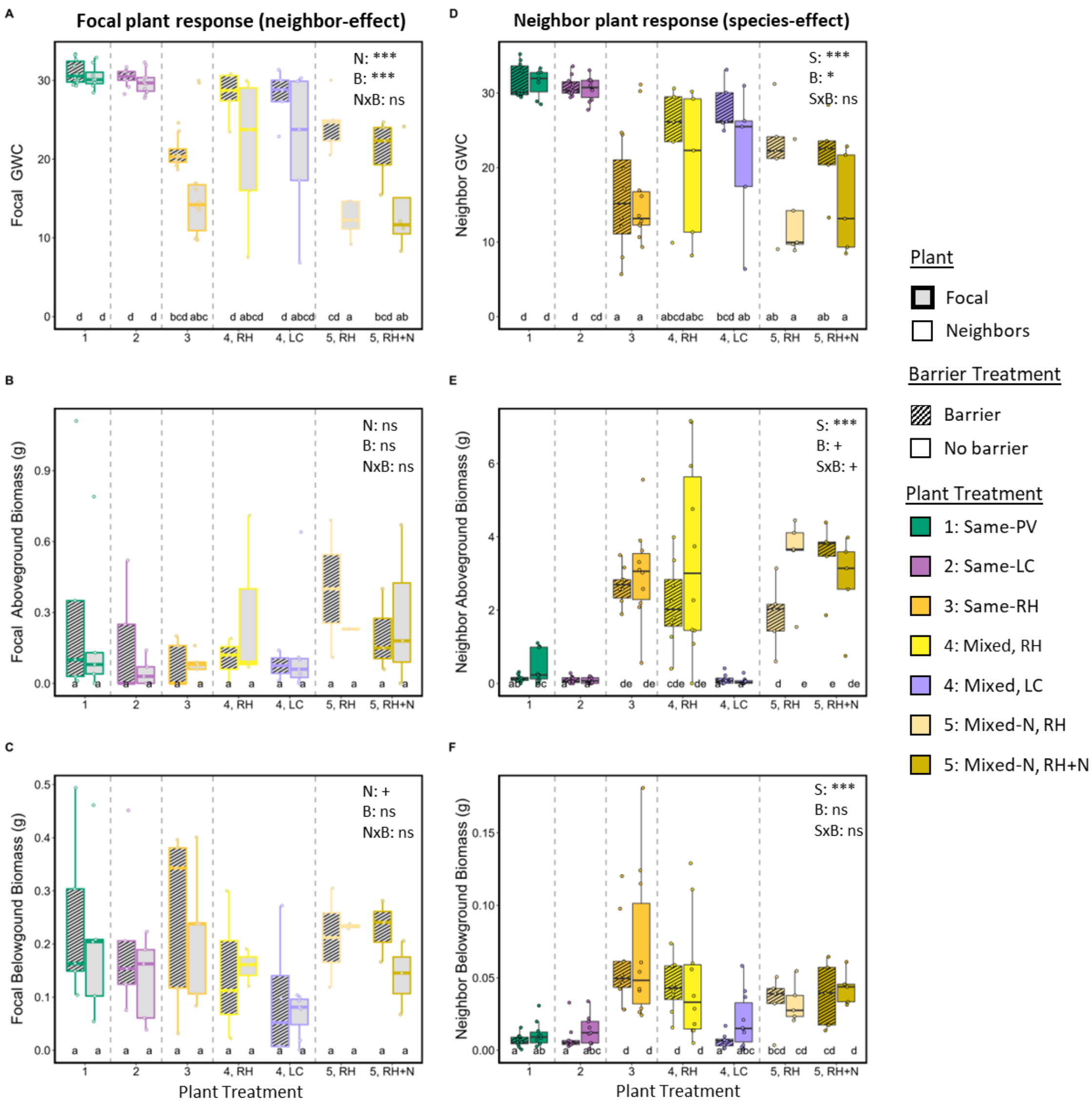

Figure 3.2. Variation in gravimetric water content (GWC, A & D), aboveground biomass (B & E), and belowground biomass (C & F) for the focal plant, *P. virgatum* (first column, ABC) and neighbor plant species (second column). Color and number denote neighbor treatment. Vertical gray lines separate samples present within a single treatment.. Striped boxes represent barrier treatment (no root interactions) and solid boxes represent the no barrier treatment (active root interactions). The central line is the median value for each plant, vertical bars represent the first and third quartiles of the data, raw data shown in points. Different letters denote significant differences among all plants and barriers (false discovery rate, $p < 0.05$). ANOVA results in upper right corner of each panel denote significant *p*-value for Neighbor (N), Barrier (B), Neighbor by Barrier interaction (NxB) for focal plants (Panels A-C) and Species (S), Barrier (B), and Species by Barrier (SxB) interaction for neighbor plants (Panels D-F); significance values: ns $p > 0.10$, + $p < 0.10$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FOCAL ROOT EXUDATES NOT IMPACTED BY NEIGHBOR IDENTITY

Neighbor identity and presence of barrier did not significantly alter the focal plant root exudate richness (Neighbor: $p = 0.087$, Barrier: $p = 0.802$), Shannon diversity (Neighbor: $p = 0.428$; Barrier: p =

0.647), or composition (Table 3.2, Supplementary Figure 3.3B). Soil moisture, as well as the aboveground biomass and shoot CN of the focal plant explained a small, but significant, portion of the variation in the root exudate profiles (RDA, overall model significance F = 3.49, p = 0.001; constrained proportion = 0.084, GWC p = 0.050, Shoot CN p = 0.002, aboveground biomass p = 0.045). Variation among the sampling days, which was treated as a conditional variable in the model, explained most of the variation in the root exudates (RDA; conditional proportion = 0.269; PERMANOVA: sampling day effect R^2 = 0.18; p < 0.001). This may have masked any treatment effects, if present.

ROOT AND RHIZOSPHERE NICHES HOST DISTINCT MICROBIOMES

The rhizosphere compartment (root-associated or rhizosphere soil) had a significant effect on the beta diversity, beta dispersion, and alpha diversity of both the fungal and bacterial microbiomes. In both instances, the root-associated communities were more variable (Beta dispersion by compartment: Fungi F = 815.2, p < 0.001; Bacteria F = 720.58, p < 0.001) and had lower Shannon diversity compared to the rhizosphere soil communities (compartment effect: Fungi F = 803.9, p < 0.0001; Bacteria F = 961.7, p < 0.0001). Even though the communities varied in diversity and composition, there was substantial overlap in the taxa that dominated each rhizosphere compartment. The fungal compartments were both dominated by the phylum Ascomycota (35% of root-associated reads; 40% of rhizosphere soil reads) and Glomeromycota were also dominant in the root-associated samples (4% of reads), while Basidiomycota (2% of reads) and Mortierellomycota (2% of reads) were more prevalent in the rhizosphere soil. On average, 85% of the fungal reads and 31% of the fungal OTUs found in the roots were present in the rhizosphere soils. For the bacteria, the most abundant phyla for both rhizosphere compartments were Proteobacteria (22% of root-associated reads; 25% of rhizosphere soil reads) and Actinobacteria (13% of root-associated reads; 25% of rhizosphere soil reads). On average, 40% of the bacterial reads and 42% of the bacterial OTUs found in the roots-associated microbiomes were also present in the rhizosphere soils.

SPECIES-SPECIFIC EFFECTS STRONG FOR ROOT, BUT NOT RHIZOSPHERE, MICROBIOMES

We found that the neighbor species associated with distinct root-associated microbiomes, but had only weak effects on the rhizosphere soil communities. This was true for both alpha diversity, where only the root-associated communities differed in alpha diversity (Supplementary Figure 3.5A,C; species effect: fungal root F = 14.83, p < 0.0001; fungal rhizosphere soil F = 1.49, p = 0.25; bacterial root F = 3.42, p = 0.02; bacterial rhizosphere soil F = 0.17, p = 0.98), as well as beta diversity (Table 3.1, Figure 3.3). Plant species explained 60% and 23% of the variation in the root-associated fungal and bacterial communities, but only 8.6% of the variation in the rhizosphere soil fungal communities and none of the variation in the rhizosphere soil bacteria (Table 3.1, Figure 3.3). Due to previous observations of

Glomeromycota in neighborhood studies (Mummey et al., 2005; Hausmann and Hawkes, 2009; Horn et al., 2017), and the known importance of arbuscular mycorrhizal fungi on plant communities, we evaluated if their composition and relative abundance responded to species and neighbor effects (reported below). Host species did significantly affect the abundance and composition of Glomeromycota found in the root-associated communities (relative abundance ANOVA: F = 4.03, p = 0.006; composition PERMANOVA Species effect: $p < 0.001$, $R^2 = 0.072$), with an overall higher abundance in the roots of the RH neighbor species than in the roots of the LC and PV neighbor species(17% of reads in RH vs. 5% of reads in LC and PV).

For a more detailed comparison of the species-specific effects on microbiome assembly, we investigated the microbiomes' Bray-Curtis distance within and among all species in the same rhizobox (see sampling design, Figure 3.1). We found that root-associated communities were most dissimilar when collected from two different host species (inter-species), followed by samples from different plants of the same species (intra-species), and samples from within the same plant (intra-pant) were the most similar (Figure 3.4A,C). This pattern was generally consistent for the root-associated bacteria and fungi, but there was greater intra-species variation for the fungal than bacterial root communities (light gray bars in Figure 3.4A vs. 4C). Comparing the two rhizosphere compartments, we found that within a single plant (intra-plant) the rhizosphere soil microbiomes were more variable than the root-associated microbiomes. In fact, rhizosphere soil microbial communities collected within the same focal plant (n = 2) were as different from each other as two rhizosphere soil communities collected from different plant species (n = 2, intra-plant versus inter-species, Figure 3.4B & 4D).

Table 3.1. PERMANOVA results showing effect of plant species and barrier effect on root-associated and rhizosphere soil microbiomes. Significant effects bolded ($p < 0.05$) and R^2 value only reported for significant effects.

Neighbor Plant Responses (Species-Effect)								
	Root-associated Fungi		Rhizosphere Soil Fungi		Root-associated Bacteria		Rhizosphere soil Bacteria	
	F	p, R^2	F	p, R^2	F	p, R^2	F	p, R^2
Species-effect	19.35	0.001, 0.60	1.21	0.025, 0.09	4.02	0.001, 0.24	0.99	0.47
Barrier	1.79	0.01, 0.01	1.04	0.39	1.06	0.33	0.97	0.52
Species*Barrier	0.80	0.96	0.87	0.95	0.77	1	0.89	0.95

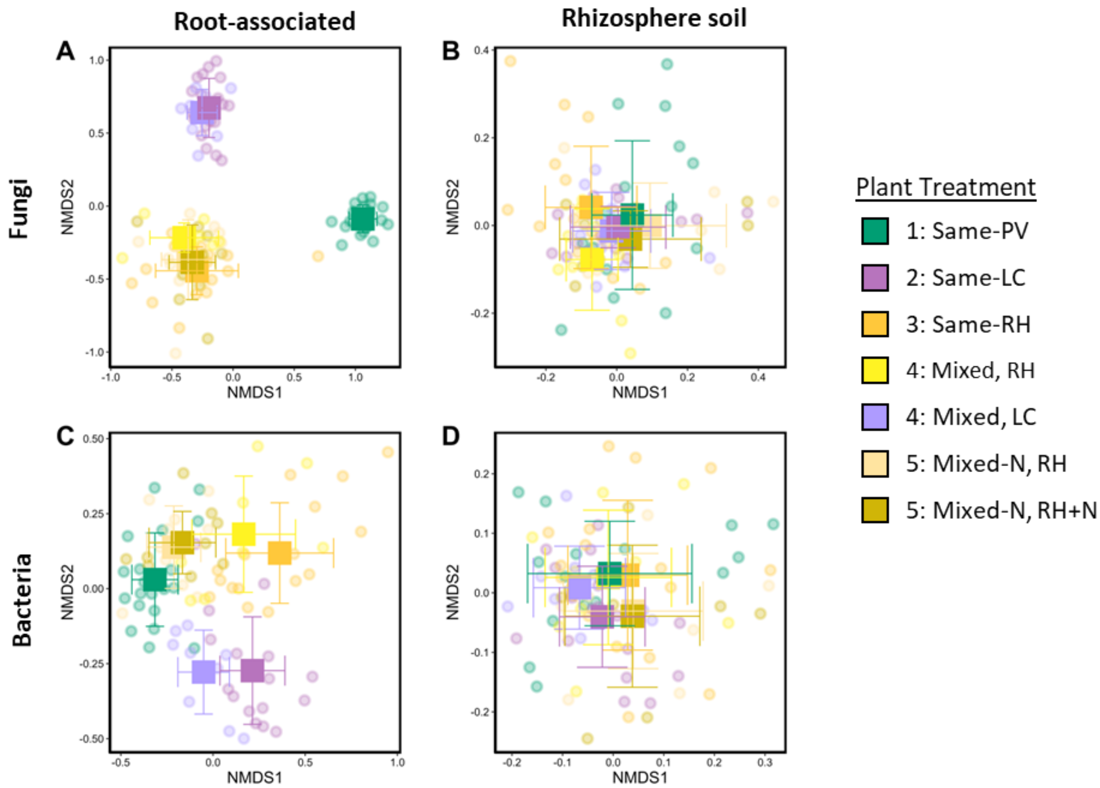

Figure 3.3. NMDS ordinations (Bray-Curtis) of neighbor plant microbiomes for root-associated fungi (A), rhizosphere soil fungi (B), root-associated bacteria (C), and rhizosphere soil bacteria (D). Neighbor plants are colored by their species and treatments. Barrier and No Barrier samples are combined within each treatment. Large squares represent centroid of all sample points and bars represent ± 1 SD from the centroid mean. See Table 3.1 for neighbor plant statistics. See Table 3.2 and Figure 3.S6 for focal plant microbiome statistics and NMDS ordinations.

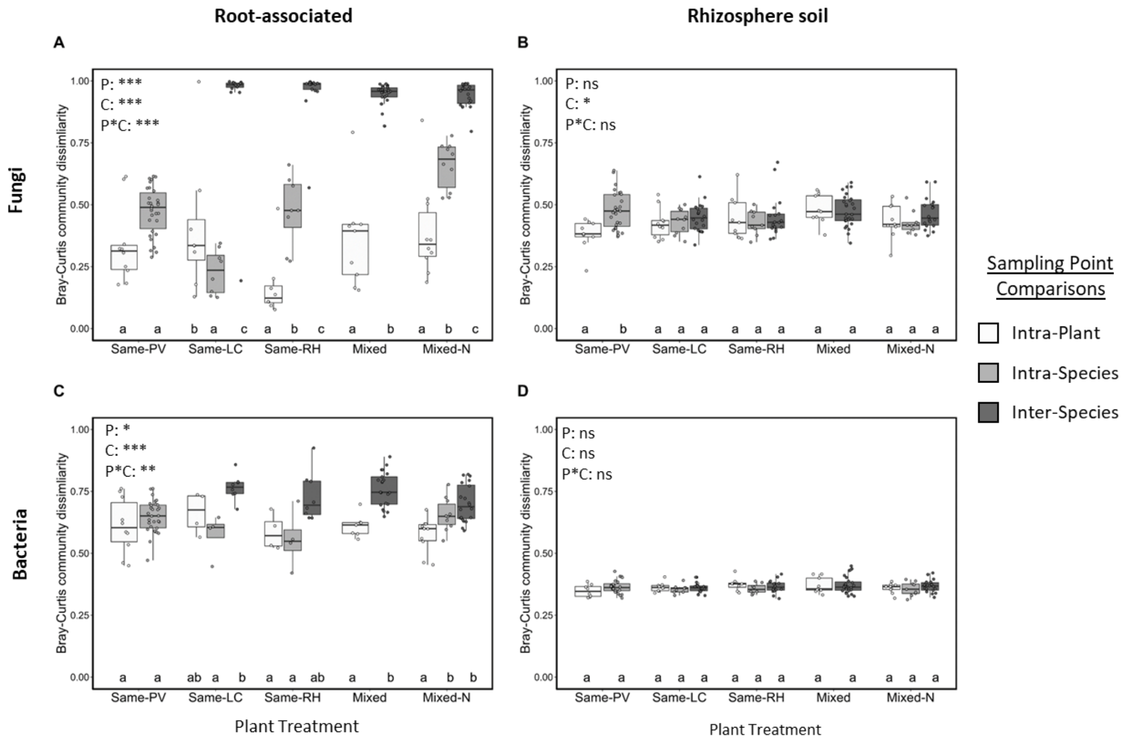

Figure 3.4. Microbiome community dissimilarity (Bray-Curtis) between plants in a single rhizobox for root-associated fungi (A), rhizosphere soil fungi (B), root-associated bacteria (C), and rhizosphere soil bacteria (D). Shaded bars represent comparisons among the sampling points (see shaded circles in Fig. 1 for sampling points); Intra-plant compares two samples within the same focal plant; Intra-Species compares samples collected from different plants of the same species; Inter-Species compares two samples collected from different species. Lower dissimilarity values indicate the communities are more similar, larger values indicate they are more different. The central line is the median value for each plant, vertical bars represent the first and third quartiles of the data, raw data shown in points. Barrier and No Barrier treatments are combined because they did not significantly differ in the models. ANOVA results in upper right corner for Plant Treatment (P) and sampling point comparisons (C); significance values: ns $p > 0.10$, + $p < 0.10$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Different letters denote significant differences among Comparisons within each Plant Treatment (false discovery rate, $p < 0.05$).

NEIGHBOR PLANTS IMPACT FOCAL PLANT ROOT-ASSOCIATED BUT NOT RHIZOSPHERE SOIL MICROBIOMES

Consistent with the microbiome responses to plant species, we found that plant neighbors altered the composition and diversity of the focal plant root-associated, but not rhizosphere soil, microbiomes. Neighbors had significant, though subtle, effects on the Shannon diversity of the focal plant's root-associated fungal community, but not on the root-associated bacterial or either rhizosphere soil community (Supplementary Figure 3.5B,D; Neighbor effect: root fungi F = 8.83 , $p < 0.0001$; rhizosphere soil fungi F = 1.34, $p = 0.30$; root bacteria F = 6.14, $p = 0.16$, rhizosphere soil bacteria F = 1.63, $p = 0.21$). The composition of the focal plant's root-associated microbiomes was also impacted by neighbor (Table 3.2, Supplementary Figure 3.6), and this neighborhood effect was influenced by differences in beta dispersion (Neighbor effect Betadispersion: root fungi F = 3.39, $p = 0.002$; root bacteria F = 4.51, $p = 0.006$), as well as by the presence of barrier for the fungal communities (significant neighbor * barrier interaction, Table 3.2). The focal plant root-associated fungal communities were most different from each other when grown next to the Same-RH and Same-PV neighbor treatments, but only when there was no barrier (pairwise PERMANOVA, $p = 0.004$). Several fungal genera contributed to this difference, including enrichment of *Paraglomus* (Phylum: Glomeromycota) and reduction of *Hannaella* (Phylum: Basidiomycota) when the focal plant neighbored PV versus RH (AncomBC results, $p < 1.2\ e^{-4}$). Unlike the overall fungal community, neighbor did not impact the composition of Glomeromycota within the focal plant (PERMANOVA F = 0.94, $p = 0.76$), but did influence the relative abundance (neighbor effect F = 3.95, $p = 0.007$). On average, Glomeromycota made up 4.3% of reads in the focal root microbiome, but this was slightly higher when neighbored by PV (treatment 1) or RH in the mixed treatment (treatment 4) (Figure 3.S7).

Table 3.2. PERMANOVA results showing effect of neighbor treatments (merged value based on neighbor treatment and species identity) on focal plant root-associated and rhizosphere soil microbiomes and root exudates. Significant effects bolded ($p < 0.05$) and R^2 value only reported for significant effects.

Focal Plant Responses (Neighbor-Effect)										
	Root-associated Fungi		Rhizosphere soil Fungi		Root-associated Bacteria		Rhizosphere soil Bacteria		Root exudates	
	F	p, R^2	F	p, R^2	F	p, R^2	F	p, R^2	F	p, R^2
Neighbor-effect	1.47	0.01, 0.10	1.01	0.44	1.62	0.007, 0.09	0.94	0.83	1.39	0.10
Barrier	1.83	0.033, 0.02	1.35	0.067, 0.02	1.09	0.27	1.17	0.13	0.41	0.74
Neighbor*Barrier	1.29	0.001, 0.09	0.92	0.806	0.89	0.92	0.96	0.68	0.84	0.68

HOST PLANT GROWTH, SOIL CONDITIONS, AND ROOT OVERLAP CONTRIBUTE TO NEIGHBOR-EFFECTS

The barrier treatment, which prevented active root interactions, had insignificant to weak effects on microbiome composition for the focal and neighbor plants (neighbors: Table 3.1; focal: Table 3.2). There was a weak, though significant interaction between barrier and neighbor on the focal root-associated fungal communities (Table 3.1), but there were no significant pairwise interactions for barrier across treatments. For all microbiomes, the presence of a root barrier also did not affect the percentage of reads shared between the focal and neighbor plants (Supplementary Figure 3.8). In fact, averaged across all treatments, over half of the reads from the focal plant's root-associated and rhizosphere soil communities were shared with the direct plant neighbor, regardless of barrier treatment (root fungi: 64%; rhizosphere soil fungi: 86%; root bacteria: 50%; rhizosphere soil bacteria: 80%).

We also found that host plant growth and nutritional status, and soil factors contributed to the composition of the focal plant's microbiomes. The structure of the fungal, but not bacterial, communities was correlated with the composition of the focal plant root exudates (Protest analysis; rhizosphere soil fungi $p < 0.001$, r = 0.288, root fungi $p = 0.042$, r = 0.197; rhizosphere soil bacteria p = 0.30, root bacteria $p = 0.08$, r = 0.19). A small, but significant, proportion of variation in the focal plant's rhizosphere soil communities was also explained by soil moisture, while plant aboveground biomass and shoot CN contributed to variation in the root-associated microbiomes (Table 3.3).

Table 3.3 Distance-based Redundancy Analysis Results (Bray-Curtis) for microbiomes, showing contribution of abiotic and plant factors on microbiome structure. Replicate included as a conditional factor in analysis. 'NA' is used when the factor was not included in the model after model selection. Constrained proportion of variance signifies how much variance in the community composition can be explained by the factors.

	Root-associated Fungi	Rhizosphere soil Fungi	Root-associated Bacteria	Rhizosphere soil Bacteria
Gravimetric Water Content	NA	$p = 0.003$, $R^2 = 0.02$	$p = 0.028$; $R^2 = 0.02$	$p = 0.01$; $R^2 = 0.02$
Aboveground Biomass	$p = 0.012$; $R^2 = 0.03$	NA	$p = 0.021$; $R^2 = 0.02$	NA
Shoot CN	$p = 0.007$; $R^2 = 0.04$	NA	NA	NA
Constrained Proportion of Variance	0.068	0.020	0.038	0.016

DISCUSSION

PLANT SPECIES AND NEIGHBORS AFFECT ROOT-ASSOCIATED MICROBIOMES, BUT HAVE LIMITED EFFECT ON RHIZOSPHERE SOIL MICROBIOMES

In support of our first hypothesis, we observed stronger species effects for the root-associated than rhizosphere soil microbiome communities. The root-associated communities were also less diverse and, together, these results support previous studies demonstrating that the root is a more selective niche compared to the rhizosphere (Berg et al., 2014; Edwards et al., 2015b; Qian et al., 2019). These species-effects on the root-associated microbiomes were stronger for the fungal than bacterial communities, which is also consistent with what we observed for the rhizosphere soil communities. The bacterial soil communities did not differ among plant species, and because the rhizosphere soil is the primary recruitment pool for the root microbiome, this likely contributed to the weaker species-effect on the bacterial root communities. Other studies also report that soil fungi respond more strongly to their host plant than soil bacteria, perhaps because bacteria are more sensitive to changes in abiotic conditions that are unrelated to their host plant (Urbanová et al., 2015; Emilia Hannula et al., 2019).

Similar to the stronger species-effects on the root-associated microbiomes, and in partial support of our second hypothesis, we only observed neighborhood effects on the focal plant's root microbiomes (but not rhizosphere soil), and the overall effect was weak. Previous work supports that mycorrhizal taxa are particularly responsive to changes in plant neighborhoods (Mummey et al., 2005; Hausmann and Hawkes, 2009; Morris et al., 2013) and while one arbuscular mycorrhizal fungal (AMF) genus (*Paraglomus*) differed among the neighbor treatments, the overall AMF composition did not. Even though we found that the AMF communities differed among plant species (explaining 7% of the variation), studies show that most AMF taxa are often plant generalists (Klironomos, 2000; Öpik et al., 2010), so perhaps less influenced by neighborhood, as shown in another field study (Horn et al., 2017). The lack of neighborhood effect on the rhizosphere soil communities is surprising, as these have been reported previously (Hortal et al., 2017b; Cavalieri et al., 2020; Chen et al., 2020), including in our previous study that used the same plant species (Ulbrich et al., 2022). However, in those studies, the neighbor and focal plant species had distinct rhizosphere soil communities, and it is plausible that the limited species-effects we observed on the rhizosphere soil communities may have influenced the potential for neighborhood effects to occur (discussed more below).

We suggest that unique properties of our experiment, including our use of field soil and fine-scale sampling, likely contributed to the weak species and neighborhood effects on rhizosphere soil communities. The use of field soil in the rhizoboxes brought our experiment one step closer to natural conditions, but subtle shifts in active taxa may have been masked by DNA from dead or dormant cells (Vályi et al., 2016; Carini et al., 2017; Gkarmiri et al., 2017; Runte et al., 2021). Other neighborhood studies that use DNA-based methods may not encounter this challenge because they either focus on a specific group of taxa in field settings, such as AMF (Mummey et al., 2005; Morris et al., 2013; Horn et al., 2017) or, in greenhouse settings, use only a small percent of field soil to inoculate potting soil (Hausmann and Hawkes, 2009; Cavalieri et al., 2020; Mony et al., 2021; Ulbrich et al., 2022). It is also possible that species-specific conditioning of rhizosphere soil communities takes longer to appear in field soils than in inoculated substrates. In fact, a recent study found that seedlings transplanted into a mature grassland did not assemble distinct fungal or bacterial soil communities even after six months (Schöps et al., 2018), while in inoculated greenhouse soils this can occur after only several months (Cavalieri et al., 2020; Ulbrich et al., 2022). Together, these studies suggest that the use of 100% field soil and three-month growing period contributed to the weak species- and neighborhood-effects observed here.

A second factor that may have contributed to the extreme heterogeneity in the rhizosphere soil microbiomes is our fine-scale sampling approach. We used this approach to improve our ability to detect localized neighbor responses, which we did not observe, and it may have inflated the microbial beta diversity within a single plant. Previous studies show that rhizosphere soil bacterial and fungal communities can vary along a single root, as well as across different types of roots (Kawasaki et al., 2016; Yu et al., 2018; Rüger et al., 2021). Although we targeted similar looking roots in the focal plant, it is possible that subtle differences in roots contributed to the high heterogeneity. In fact, consistent with the patterns we observed, microbiomes from different root types (e.g. perhaps the two root samples collected from the focal plant) can be more dissimilar than communities from different plant species (Figure 3.4) (Wei et al., 2021). This may suggest that stochastic processes play a large role in fine-scale assembly of rhizosphere soil microbiomes, but similarly could also indicate that the host plant has fine-scale, localized control on microbiome assembly. While there are benefits to this fine-scale sampling approach, such as detecting changes in particular OTUs (e.g. after localized inoculation), sampling from the entire root system results in a more consistent representation of a plant's microbiome (Wei et al., 2021). Therefore, it may not be surprising that we did not observe neighborhood or species effects with our fine-scale sampling, especially when dealing with a diverse soil community and relatively low sample sizes.

MECHANISMS MEDIATING NEIGHBORHOOD EFFECTS ON MICROBIOME ASSEMBLY DIFFER FOR ROOT AND RHIZOSPHERE

We hypothesized that neighborhood effects on the root-associated microbiomes would be mediated primarily by the host-plant, while the rhizosphere soil communities would be more strongly impacted by the spillover of novel neighbor microbes during active root interactions. However, because the plant species did not assemble distinct rhizosphere soil communities, it is unlikely that active root interactions would lead to compositional shifts, even if spillover did occur. Therefore, the observed neighbor-induced shifts in the focal plant's root-associated microbiomes – even in the absence of neighbor effects on the rhizosphere soil communities– indicates that the host plant played a strong role in mediating neighbor effects on the root-associated communities. We did not observe neighborhood effects on the focal plant biomass, tissue CN, or root exudates, suggesting that other mechanisms, such as neighbor-induced changes in focal plant gene expression (Bowsher et al., 2017; Liao et al., 2021) or volatile signaling (Li et al., 2020; Kong et al., 2021) may have altered the focal plant's root-associated microbiome. Unlike the root-associated microbiomes, the plant neighbors did not impact the focal plant rhizosphere soil communities. However, we predict this is due to the lack of variation in the species' microbiomes, rather than the absence of microbial spillover. In fact, in our previous study, a focal plant (also PV) and an RH neighbor assembled distinct rhizosphere soil bacterial communities when grown alone, but when grown together there was evidence of taxa specific to RH increasing in abundance in the focal plant rhizosphere soil (Ulbrich et al. 2022). Together, these results suggest that the introduction of novel microbes during active root interactions plays a large role in mediating neighborhood effects on rhizosphere soil communities, but that these effects are contingent upon the plant species harboring distinct microbiomes in the first place. A second mechanism influencing rhizosphere soil community assembly in this study was neighbor-induced changes in soil moisture.

Across all treatments, focal plant soil moisture was lower when there were active root interactions (no barrier), and this was exacerbated when there were two RH neighbors. This variation in soil moisture had a small, though significant, effect on the fungal and bacterial rhizosphere soil communities, the bacterial root community, and root exudates. It was still not enough to drive treatment effects, perhaps because the soil moisture differences did not affect focal plant biomass. Overall, it is clear that soil moisture contributed to shifts in microbiome assembly, and in a longer study may have contributed to stronger neighborhood effects, but it is unclear if these changes were mediated by the host-plant, such as through changes in root exudates (Gargallo-Garriga et al., 2018), or through direct responses to soil moisture (Kaisermann et al., 2015; Naylor and Coleman-Derr, 2018).

Finally, though we did not observe neighbor-induced shifts in root exudates, which further corroborates the lack of neighbor-effect on the rhizosphere soil communities, we did observe significant correlations between the focal plant root exudates and the fungal communities. This supports previous studies showing that root exudates impact microbiome assembly (Steinkellner et al., 2007; Broeckling et al., 2008; Hugoni et al., 2018), but it is also possible that these correlations are not reflective of the root exudates mediating fungal assembly, but rather of the fungal communities altering the plant's root exudates (Vos et al., 2012; Guo et al., 2015) or of fungal-derived metabolites present during our non-sterile root exudate collection . We were surprised that the bacterial communities did not correlate with the root exudates, as bacterial rhizosphere assembly has previously been shown to be regulated by root exudates (Zhalnina et al., 2018). It may be that variation in the root exudates across sampling days masked any potential relationship between the root exudates and soil bacteria. Overall, these data reveal that while root exudates and microbiomes may be correlated, neighbor-induced shifts in exudates are not the primary driver of neighbor-effects on microbiome assembly.

CONCLUSION

In summary, we found neighbor-induced changes in root-associated but not rhizosphere soil microbiomes, and that these neighbor effects are mediated by the host-plant, at least in the absence of neighbor effects on rhizosphere soil microbiomes, which could alter root recruitment. We saw surprisingly little plant species-specific effects on rhizosphere soil microbiomes, and no significant neighbor effects, but neighbor-induced shifts in soil moisture had significant but subtle effects on rhizosphere soil community assembly. Together, these results may indicate that neighborhood effects on rhizosphere soil microbiomes occur first through subtle shifts in abiotic conditions, but that larger compositional shifts depends on the introduction of novel neighbor microbes during active root interactions. Lastly, we observed substantial heterogeneity among rhizosphere soil samples collected on individual roots, even within the same plant. This may indicate some localized control from the host plant, but also suggests that samples collected from the entire root system may be more representative of overall neighborhood effects on rhizosphere assembly. Overall we show that host-plants play a role in mediating neighborhood effects on root microbiomes, but neighborhood effects on rhizosphere microbiomes depend upon each species harboring a distinct microbiome in the first place.

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CHAPTER FOUR: PERSPECTIVES AND INSIGHTS ON SOIL HEALTH FROM MICHIGAN FARMERS

ABSTRACT

The potential for healthy soils to address goals of productivity and sustainability has motivated a global soil health movement. Though there are many interested groups, understanding farmers' perceptions of soil health is essential for making research and outreach efforts applicable on-farm. Most studies investigating farmers' perceptions either focus on a specific practice, rather than soil health more broadly, or use a single method, which makes it challenging to describe both the breadth and depth of farmers' engagement with soil health. To address this gap, we use surveys, interviews, and mental models to describe how Michigan farmers understand, evaluate, and manage for soil health. We found that Michigan farmers believe in the benefits of soil health and identify many properties that impact it, but they also describe challenges with evaluating and managing for soil health. Farmers primarily used traditional agronomic soil tests, yield, and qualitative indicators (e.g., yield, crop coloration, soil texture) to evaluate differences in soil health. However, several farmers also described how comparisons across soil types can be problematic and, thus, that there is a need for regionally-defined soil health benchmarks. Regarding management, we found that water stress and pest pressure are top concerns and that describing the potential for soil health to mitigate these concerns could help farmers relate soil health to their management decisions. Finally, we show that many Michigan farmers believe they are taking steps to improve the health of the soils they farm, which was reflected in their use of no-till and cover crops, as well as other practices. Overall, this study emphasizes that Michigan farmers have a deep understanding of soil health and that they are taking steps to improve it, but that there are still many research and outreach efforts which could help farmers more intentionally fit soil health into their management decisions.

INTRODUCTION

"I'm trying to do everything I can to make this ground produce what it can for me and my kids and my grandkids. Soil health is obviously a big part of that. So I try to actively do what I can to maintain or improve where I can. But to say that I've exhausted every effort to improve just soil health, I haven't gotten there yet." [MI3W104]

Farmers across the globe work to balance goals of productivity and sustainability, and soils are often at the center of this balancing act. Healthy soils can support farmers' economic and environmental goals (Oldfield et al., 2019), as well as aid in their ability to respond to and mitigate climate change (Lal, 2004; Hewitt et al., 2021). This potential power of soils has given rise to a global soil health movement, with increased interest and investment from academic institutions, governments, and private businesses. Defined broadly as "the continued capacity of soil to function as a vital living ecosystem that sustains plants, animals, and humans” (USDA-NRCS), soil health has been able to unite many groups (Lehmann et al., 2020; Powlson, 2020), but it also has its challenges (Janzen et al., 2021). For instance, groups develop their own definition and perception of soil health, challenging communication across relevant groups. Farmers have the ultimate power to improve the health of agricultural soils and, therefore, it is essential that their perceptions of soil health are both acknowledged and used to inform research and outreach efforts.

Most previous work describing farmers’ views on soil focus on their use of conservation practices (e.g., no-till, cover crops) (Carlisle, 2016; Prokopy et al., 2019; Ranjan et al., 2019), rather than how they conceptualize soil health itself. While these studies show that soil health is often central to farmers’ decisions to adopt conservation practices, understanding how farmers perceive soil health may inform what soil properties are more likely to guide farmers’ management priorities. The few studies that have done this describe farmers’ soil health knowledge as holistic, practical, and informed by years of experience. For instance, farmers often relate soil health to their yield goals (Andrews et al., 2003; Roesch-McNally et al., 2018; Bagnall et al., 2020) and evaluate their soils in qualitative terms, like how it smells or how well it works up with a tractor (Romig et al., 1995).

Connecting farmers’ soil health knowledge to their management decisions can be challenging, but multiple methods can help describe the complexity (Jick, 1979; Olsen, 2004). For instance, a study that used qualitative interviews coupled with quantitative lab-based assessments found farmers’ identification of their “best” and “worst” fields correlated with several quantitative indicators of soil health (O’Neill et al., 2021). By using two methods, this study found that farmers’ intuitive sense of soil health could be used to guide management, as well as the development of more practical, farmer-informed indicators. Another example of this mixed-method approach, referred to as triangulation, includes the use of surveys and interviews, whereby researchers can broadly characterize a populations’ knowledge or behaviors, and then, through interviews, inform *how* and *why* individuals make certain decisions. In agricultural research, there is ample data about farmers’ management decisions (e.g., USDA-NASS Census of Agriculture; USDA-ARMS), but only more in-depth, qualitative studies can reveal the nuances of farmers’ actions and how they relate to soil health (Bagnall et al., 2020; Friedrichsen et al., 2021; Irvine et al., 2023).

Another less common, though effective way to more deeply understand farmers’ perceptions is through mental models (Halbrendt et al., 2014; Hoffman et al., 2014; Bardenhagen et al., 2020; Friedrichsen et al., 2021). Mental models represent an individual’s unique experiences, values, and beliefs that inform their decisions and actions (Johnson-Laird, 1983; Jones et al., 2011), and provide a unique framework to identify what priorities and values guide farmers' thought processes and decisions. For instance, previous studies found that when farmers' mental models included more conservation-related concepts, they were also more likely to use conservation-friendly management practices (Vuillot et al., 2016; Bardenhagen et al., 2020). It is probable, then, that farmers' mental models of soil health may also reflect their preferred soil health management practices, as well as what indicators of soil health they are most comfortable using (Lobry De Bruyn and Abbey, 2003). In fact, outreach programs that consider farmers' mental models in their communication efforts are more likely to have their advice implemented on-farm (Eckert and Bell, 2005; Hoffman et al., 2014). Therefore, by coupling analyses of farmers' mental models with in-depth interviews, we can get a better sense of how farmers currently understand and manage for soil health, as well as how their insights can inform soil health research and outreach efforts.

To this end, we use a mixed-methods approach that includes surveys, interviews, and mental models, to answer three primary questions: 1) How do farmers understand soil health?, 2) How do farmers evaluate soil health? And 3), How do farmers' perceptions of soil health inform their management decisions?

METHODS

STUDY CONTEXT

Farmers' knowledge and management decisions are informed by local context, so this study focuses specifically on row crop farmers in Michigan, a state in the northcentral region of the United States. Row crops make up 68% of Michigan's harvested land, and include corn (31%), soy (29%) and wheat (8%) (2021 USDA-NASS Michigan Agriculture Overview). Several factors make farming unique in Michigan compared to other Midwestern states (e.g. Illinois, Indiana, Iowa), including the diversity of other crops planted in the region, shorter growing seasons and close proximity to the Great Lakes (USDA-NASS Census of Agriculture). Michigan soils are less fertile than those in other midwestern states, and have more varied soil types (Staff, n.d.). Across the state, there are initiatives to help farmers improve their soil health(e.g. Michigan Agriculture Environmental Assurance Program, MAEAP). This context likely contributes' to farmers' management decisions and their understanding of soil health.

DATA COLLECTION & ANALYSIS

We used survey and mental model approaches to evaluate how Michigan farmers understand, evaluate, and mange for soil health. First, we conducted a survey across the state to assess trends in farmers' knowledge about soil health, as well as their adoption of soil health practices. Then, using a nested-design, we contacted a subset of these farmers to participate in follow-up mental modeling activities and interviews.

SURVEY

The Panel Farmer Survey is a self-administered mail survey targeted at row crop farmers in the Midwest, and has been conducted annually since 2017, with repeated and new participants surveyed each year.[3] The survey questionnaire was developed by a multidisciplinary group of scientists and included 20 pages of questions on a range of topics including demographics, farm operation characteristics, management practices, soil health, and challenges facing agriculture. In this paper, we use only responses from the 2019 survey of Michigan farmers (response rate 46.8%), focusing specifically on their use of conservation practices, as well as their perceived knowledge about and confidence in soil health. Each question was asked with a 5-point Likert scale rating. Several questions regarding farmers' perceptions of soil health, including whether they had "taken steps to improve the health of soils they farm" were adopted from the 2015 Iowa Farm Poll Survey (Arbuckle, 2016). Demographic data (age, sex, education, farm size) were collected in each year of the survey, though these data were coalesced from previous years if there was no response in the 2019 survey.

Survey responses were analyzed for farmers operating at least 100 acres (40.5 ha), which included 353 total responses. Data were cleaned, sorted, analyzed, and plotted using R statistical software (Team, 2022) and the following packages: dplyr (Wickham et al., 2022), reshape2 (Wickham, 2007), ggplot2 (Wickham, 2016), likert (Bryer and Speerschneider, 2016). Data about the participants' use of management practices were coalesced from survey and interviews, but in cases where the survey and interview responses differed (likely because they were conducted two years apart), we relied on the more recently collected interview data. Relationships between the farmers' knowledge of soil health and their use of cover crops was determined using a Spearman's Correlation with the "cor.test" function in R.

INTERVIEWS & MENTAL MODEL ACTIVITY

We used mental modeling activities and interviews to gain deeper insight into farmers' perceptions of soil health. Using a geospatial, purposive sampling strategy, we identified a subset of

[3] The survey was mailed to corn and soybean farmers with operations over 100 acres (40 ha) in four Midwestern states, including Illinois, Indiana, Ohio, and Michigan. USDA-NASS Census of Agriculture was referenced to ensure representative sampling based on farm size (operations less than or greater than 500 acres (204 ha). It has been mailed annually since 2017 using a modified Dillman Tailored Design Method (Dillman et al., 2014) with grower addresses purchased from a private vendor. To account for attrition, we used two-part panel design.

interview participants within ten southwest Michigan counties (Allegan, Berry, Van Buren, Kalamazoo, Cass, Branch, Calhoun, Kent, Ionia, Eaton, Ottawa). Individuals from these counties were contacted if they provided their email address and agreed to further contact in the survey (n = 47). Potential interviewees were then called and emailed invitations to participate in the study. We designed our interview pool to be representative of the region and, specifically, to represent a range of soil health practices (e.g. cover crops and tillage).

After initial phone conversations – in which farmers were made aware of the research goals and a $100 cash incentive for their involvement – they were mailed a packet of materials to complete independently and return. The packet included a description of the research project, a mental model activity workbook, and a pre-stamped return envelope. In the workbook, farmers were asked to articulate three goals for their farm in the next ten years, define “cash crop productivity” and “soil health”, read an example mental model diagram[4], and then complete their own mental model diagrams for “cash crop productivity” and “soil health”. Each mental model diagram included a key term – either “cash crop productivity” or “soil health” – surrounded by eight numbered boxes, in which participants wrote the factor they believe to impact either key term (Figure 4.S2). Using a modified Fuzzy Cognitive Mapping approach (Gray et al., 2014), participants indicated how the factors impact the focal concept (positive impact ‘+’, negative impact ‘-‘), and if there were relationships between the factors (drew arrows connecting factors). The main benefit to this approach is that it allows farmers to illustrate and interact with their own representation of their mental model, which is in contrast to approaches that extract mental models from texts and interviews (Halbrendt et al., 2014; Hoffman et al., 2014) (see *Methodological Considerations* in Supplemental Materials for more details). Furthermore, due to Covid19 restrictions the interviews and mental models were conducted remotely, and though this had the benefit of farmers engaging with their own mental models without researcher bias, this approach may have had several limitations.[5]

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[4] The example mental model diagram was about “car longevity” and served to illustrate the activity without biasing farmers’ views on the research topics. See Figure 4.S1.

[5] Another difference between our study and many other FCM studies is that, due to regulations during the Covid19 pandemic, participants were asked to depict their mental models independently, rather than in front of a researcher. Potential limitations of this method include making it more challenging to quickly identify converging themes among participants, as well as more time for the participants to work on their mental models. With more time, it is possible that the mental models may not represent the immediate, intuitive thoughts they have about soil health. Further, interviewing the participants several weeks after completing their mental models meant that they did not always remember their reasons for including a concept in their mental model.

The workbooks were returned to the main researcher, scanned, and then mailed back to farmers to guide the follow-up phone interviews. One researcher conducted all one-on-one phone interviews, which took place within 2-3 weeks of receiving the workbook, from March to June 2021, and the interviews lasted an average of 74 minutes. A semi-structured interview instrument guided each conversation and included questions about farm characteristics and management practices, knowledge of soil health, and detailed descriptions of their previously completed “cash crop productivity” and “soil health” mental model diagrams. One farmer that completed the workbook did not partake in the interview, resulting in 19 total interviews and 20 mental models.

All interviews were audio-recorded, transcribed using Otter.ai software (“Otter.ai,” 2022) and edited by hand, and thematically and structurally coded based upon responses to the interview questions using NVivo software (v. 1.6.1) (QSR International Pty Ltd, 2020). The factors included in each mental model were organized in Excel (Microsoft Corporation, 2018) and then thematically coded at two levels by the same researcher that conducted the interviews. First, the factors were coded into a common, unifying topic. For instance, multiple factors, including organic material, carbon content, and humus were coded into a single topic ‘organic matter’. Second, the topics were coded into four groups, including “soil property”, “crop”, “management practice”, and “external”. These groups were primarily used to visualize differences in the frequency of topics in the figures, as well as to discuss if farmers were more likely to include soil properties or management practices in their mental models. In the crop productivity mental model, the topic “diseases and pests”, which include fungal pathogens, weeds, and insects pests, was categorized as a soil property because many crop pests, including fungal pathogens and insects often originate from or spend at least part of their life cycle in soil (Adesemoye, 2018; Tooker and Hodgson, 2020). Tables 4.S1 and 4.S2 show how the factors were coded and categorized at each level.

In comparison to studies that have participants populate their mental model from a select word bank, the more free-form process used here may have greater bias from the researchers’ subjective categorization and aggregation, but it is less likely to limit the participants’ perceptions or stifle potentially interesting heterogeneity in their responses (Gray et al., 2014), so we believe it was the best approach for our objectives. The coded topics were summarized (frequency of times a term was mentioned at least once by an individual farmer) and plotted using the R statistical program (Team, 2022) with the following packages: dplyr (Wickham et al., 2022), tidyr (Wickham and Girlich, 2022), ggplot2 (Wickham, 2016). We also evaluated if the topics included in farmers’ soil health mental models differed depending on their use of cover crops (n = 12 used cover crops on at least some fields, n = 8 did not use).

RESULTS AND DISCUSSION

SURVEY SAMPLE DEMOGRAPHICS AND LAND MANAGEMENT

The survey sample (n = 353) was broadly representative of row crop farmers in Michigan. The average respondent age was 62 years and 98% of the respondents were male and 2% were female. According to the USDA-NASS Census of Agriculture (2017), Michigan farmers that operate greater than 100 acres are, on average, 55 years old 73% male. Nearly half (47%) of respondents had at least a high school diploma, 29% had at least some college, 21% had a bachelor's degree or higher and 3% did not have a high school diploma. As of 2018, the respondents managed an average of 1,184 acres, with 31% farming 100-500 acres and 69% farming over 500 acres (this number includes the land they own and rent from others).

The survey findings revealed that in 2018 over half (56%) of Michigan farmers used conservation tillage (defined as at least 30% residue) and no-till (64%) practices and almost half (46%) used winter cover crops on at least some of their fields (Figure 4.1). The practice use and demographics of the interview subset (n = 19) was representative of all Michigan row crop farmers (n = 353), except the subset did tend to have higher education and were more likely to use cover crops.

Overall, the adoption of no-till and use of cover crops in Michigan is comparable to other Midwestern states (Guo and Marquart-Pyatt, Sandra T., Robertson In Review). Furthermore, though cover crops and no-till practices are the most commonly discussed conservation practices, the interviews and survey data showed that Michigan farmers use a variety of practices that could impact soil health, such as applying manure, using biological soil amendments, adopting extended crop rotations (67% do this sometimes), or putting perennial vegetation in unstable areas (29% do this sometimes) (also see Table 4.1).

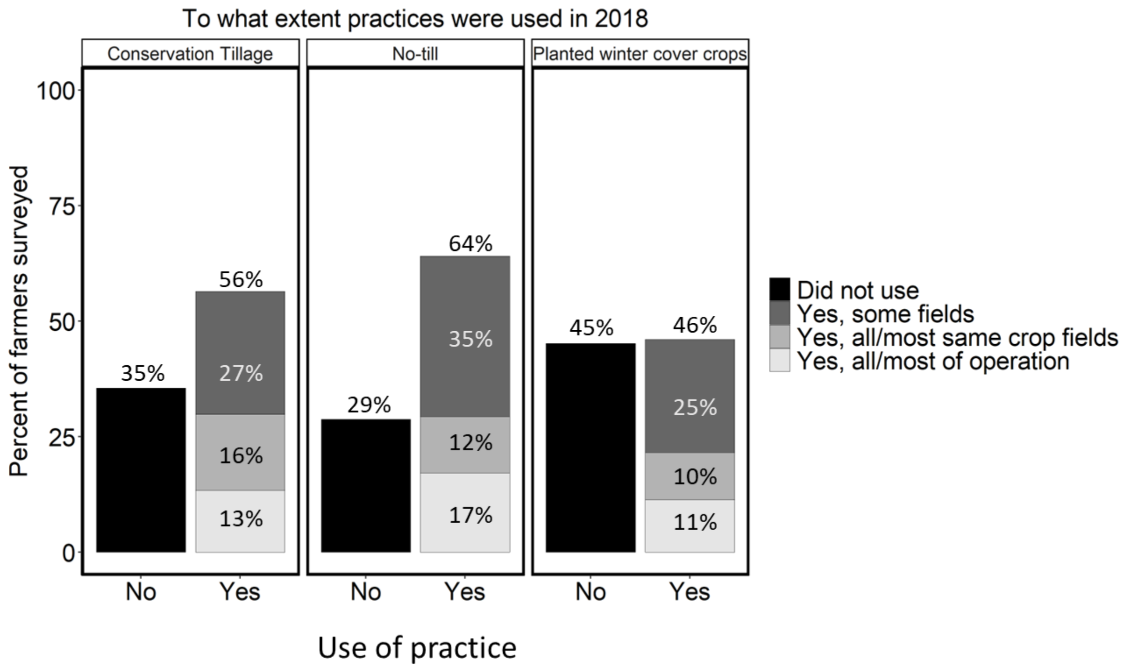

Figure 4.1. Survey responses (n ~ 320) showing percent of Michigan farmers that used soil health practices in in 2018, including A) conservation tillage (defined as > 30% residue), B) No-till, and C) winter cover crops. Five-scale likert responses to the question "To what extent did you use ____ on your farm in 2018?". Numbers do not add to 100% because the "does not reply" responses were removed.

1) HOW DO MICHIGAN FARMERS THINK ABOUT SOIL HEALTH?

1.1) FARMERS FEEL KNOWLEDGEABLE ABOUT AND BELIEVE IN THE POTENTIAL BENEFITS OF SOIL HEALTH

Our results show that Michigan farmers feel knowledgeable about soil health and believe in the benefits it can offer, but that that their past experiences do not necessarily reflect these feelings. In the survey, over half of Michigan farmers reported that they feel they know at least something about building soil health (57%) and soil organic matter (61%), and most feel knowledgeable about using soil health practices, like cover crops (44%) and no-till (51%) (Figure 4.2). Nearly all farmers reported that they believe in capacity of soil health to increase yields (98% of total sample) and drought resilience (93%) (Figure 4.3). This includes those that reported being "unsure" about their soil health knowledge (38%).[6]

[6] Of the 38% of farmers reported feeling "not sure" about their knowledge of soil health (n = 130), 98% believed that soil health increases yields, and 91% believed that soil health can increase drought resilience.

Important nuances of this seemingly-unanimous belief in the benefits of soil health were revealed through interviews. For instance, one farmer who, in the survey, shared he believed “a great deal” that healthy soils increase yields, later voiced his skepticism about this relationship: “that's my reading and research [that soil health increases crop productivity]. I'm a little bit of a skeptic. I don't necessarily believe anything ‘til we do it. But I'm also not gonna say it's not true.” [MI3W106]. If this sentiment is shared by other Michigan farmers, then it is likely that most Michigan farmers believe in the potential for healthy soils to increase yields and drought resilience, but that they will only be convinced when they see it themselves, and that surveys overestimate their belief in the potential. Other studies also show that hands-on experience is the most effective way for farmers to gain knowledge and confidence in soil health practices (Long et al., 2013; Ranjan et al., 2019). Furthermore, it has been suggested that believing that soil health can increase yields helps farmers cognitively connect their short-term productivist goals with their long-term conservation goals, perhaps even if they have not observed it themselves (Roesch-McNally et al., 2018).

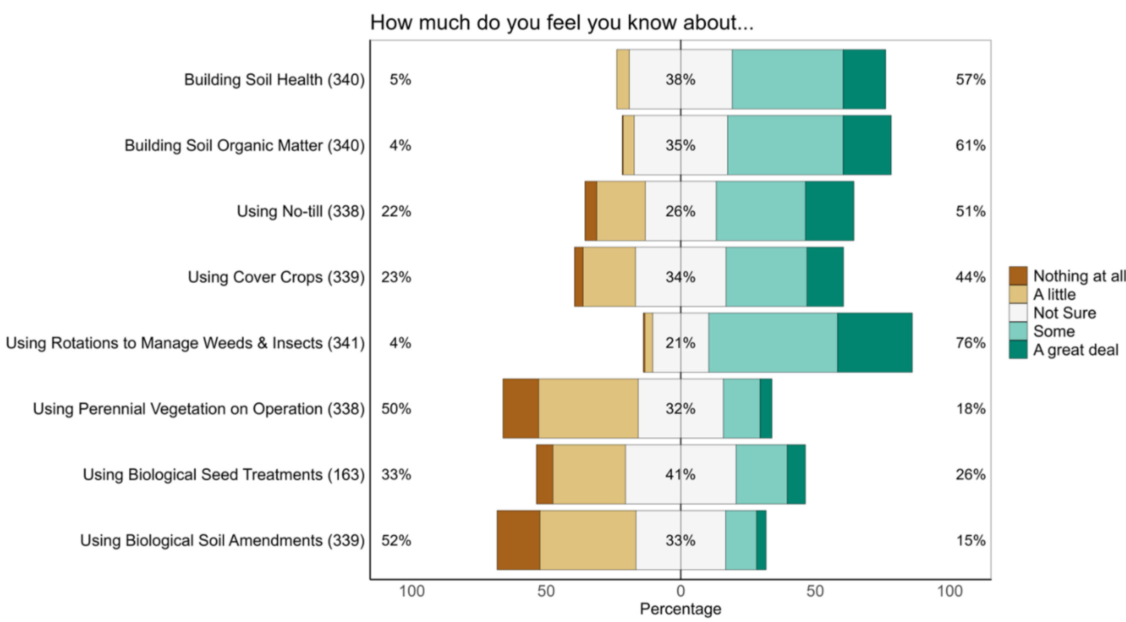

Figure 2. Survey responses for Michigan farmers’ perceived knowledge about soil health and practices. Five-scale likert responses to the question “How much do you feel you know about…?”. Number in parentheses indicates the response sample size for each variable. The question about “using biological seed treatments” was only sent to half of survey participants, which is reflected by its lower sample size.

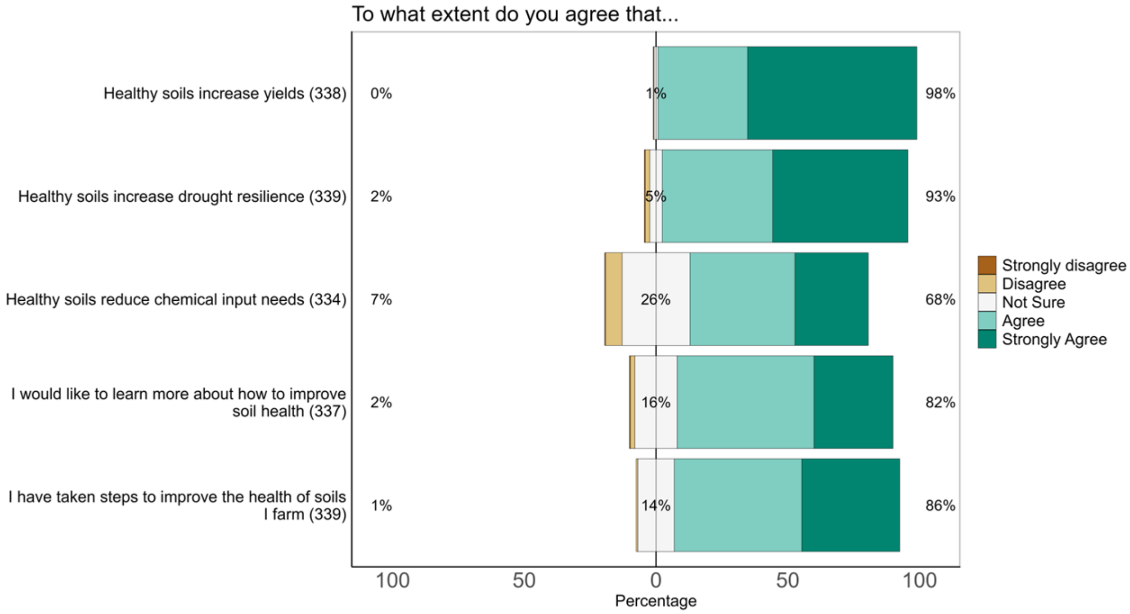

Figure 4.3. Survey responses for Michigan farmers' perceived knowledge about soil health and practices. Five-scale likert responses to the question "To what extent do you agree that…". Number in parentheses indicates the response sample size for each variable.

1.2) FARMERS HAVE A SYSTEMS-LEVEL UNDERSTANDING OF SOIL HEALTH, GUIDED BY SOIL PROPERTIES

The mental model activity and interviews revealed that farmers have a complex, systems-level understanding of soil health. We identified 30 topics that farmers think impact soil health and found that, overall, there was more agreement on which soil properties impact soil health than which management practices lead to healthy soil (Figure 4.4). In the interviews, farmers frequently described the relationships between different soil properties, as well as how the soil properties are influenced by management (Table 4.1).

The most frequently mentioned topics included organic matter (75%), compaction (65%), and soil biology (55%) (Figure 4.4, no "crop" terms mentioned in soil health mental models). Consistent with this, other studies show that farmers view increased organic matter and reduced compaction as the primary benefits of soil health practices (reviewed by (Carlisle, 2016). Soil biology has also long been valued by farmers (Romig et al., 1995). Similar to our findings, a recent study that interviewed 91 Midwestern row crop farmers found that over two-thirds of farmers discussed the importance for biological activity for soil health (Irvine et al., 2023). It could be that farmers' have a renewed focus on soil biology, as there is also increased interest in the role of soil biology for agriculture from scientists (Chaparro et al., 2012; Fierer et al., 2021), as well as private industry (Ellis, 2022). In fact, several farmers in our interviews (36%) said they are trying out new products that stimulate microbial activity in their soils, and three directly mentioned these products as a strategy to bolster the soil biology in their soils

(Table 4.1). But despite this interest, they were also uncertain about how the products work and skeptical that they might be “snake oil”, a sentiment that is consistent our survey results (Figure 4.2) and previous findings (Doll et al., 2020).

When discussing the relationships between soil health and these top three topics, farmers often described their importance for managing water, as well as how they can be impacted by cover crops and tillage (Table 4.1). They described that higher organic matter can increase water holding capacity, and that earthworms can increase water infiltration while compaction leads to ponding. Farmers also frequently discussed how cover crops and tillage play a role in managing these soil properties, as well as water-related risks. For instance, one farmer shared that: *“We try to manage too much rain with [tile] drainage, [but during] the drought, the less tillage and cover crops actually helped us.”* [MI3W111]. Other studies report that experiencing extreme weather events can motivate farmers to adopt conservation practices (Ding et al., 2009; Roesch-McNally et al., 2018). Farmers generally agreed that cover crops have a positive impact on soil health, and this was even true of farmers that do not currently use cover crops[7] (discussed more in section 3.2), but there was more variation in how tillage impacts soil health (Table 4.1).

[7] n = 2/7 farmers did not use cover crops but included it in their soil health mental model, Figure 4.4; n = 5/13 farmers did not use cover crops but play a role in increasing organic matter, see Table 4.1

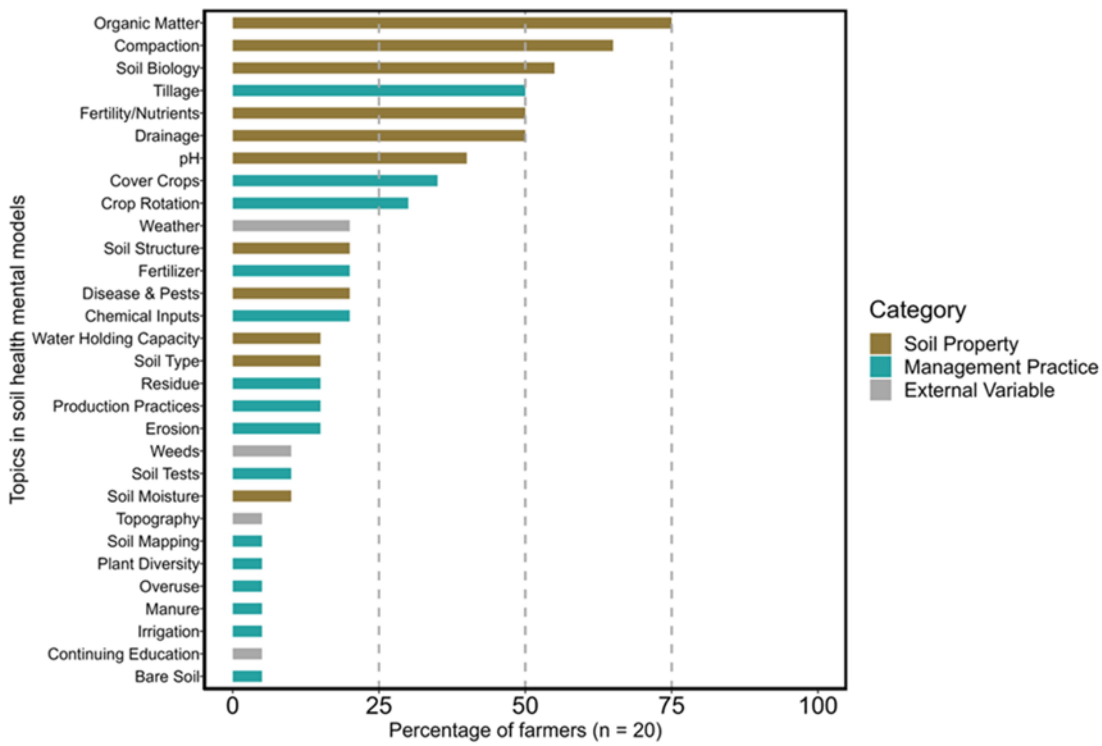

Figure 4.4. Topics included in Michigan farmers' soil health mental models, each topic consisted of several terms aggregated (Table 4.S1). Colors reflect the category of the term, as defined by the lead researcher. Gray-dashed lines indicate 25%, 50%, and 75% of farmers. The term "drainage" sometimes referred to the natural ability of the soil to hold water (soil property), but also tile drainage (management practice).

Table 4.1. Details about the most frequently mentioned topics included in farmers mental models of soil health (See Figure 4.4). Plus and minus signs indicate if farmers described the management to have a positive (+) or negative (-) impact on soil health. Numbers indicate how many farmers mentioned the concept in the interview (n_{total} = 20).

Factor	Its impact on soil health	How farmers manage it	Example Quotes: Connecting soil properties and management practices
Organic Matter	Feeds soil biology (9) Increases water holding capacity (5) and drought resilience (2) Reduces soil crusting and hardening (2) Increases crop production (1) Helps other pesticide and fertilizer applications work right (1) Reduces erosion (1) Increases fertility (1)	+ Cover Crops (13) + Leave residue on top (no-till, minimum till)(8) + Manure (2) + Incorporate residue (chisel plow) (1) + Bio-solids (1)	*"The idea is to leave as much residue on top as you can. And that will help promote earthworms, and try to build your organic matter. But if you till it.... in which a lot of farmers are still doing all the tillage, their organic matter is probably dropping and they're also losing carbon." [MI3W112]* *"Yeah, the healthier soil is, the better the crops are gonna do. So yeah, and one of the things that that really keeps soil healthy is your organic matter. And that's where cover crops help you out a lot." [MI3W102]*
Compaction	Prevents deep root growth (4) Reduces soil drainage (4) Inconsistent seed depth (2) Prevents the soil from "breathing" (1)	+ Cover crops (6) + Not driving when the field is wet (difficult with lots of ground to cover) (5) + Chisel-plowing/minimum till (4) + Equipment considerations (larger boom, larger tires, tracks on combine) (4) + Sub-soiling (2)	*"Get a cover crop down there and get your roots down and loosen the soil up…it's kind of a cure for [compaction] maybe...[cover crops] will make it pretty mellow when you work it the next year." [MI3W118]* *"Soil compaction is becoming a big deal with this bigger equipment, heavier equipment, people working more and more ground and want to get in when it's too wet. You know, all that is just really starting to have an effect. That's why I tried to plant the radishes" [MI3W116]*

Table 4.1 (cont'd)

Factor	Its impact on soil health	How farmers manage it	Example Quotes: Connecting soil properties and management practices
Soil Biology	Release nutrients and increase soil fertility (5) Build organic matter (4) Increase water infiltration (4) Reduce soil crusting (1) Worms loosen the soil (1) Help plants grow (1)	- Tillage (destroys earthworms, creates a hardpan, removes surface residue) (5) + Cover crops (provide food, aeration, macropores) (4) + Adding biologicals and soil amendments to stimulate microbial activity (3) - Fertilizers kill bacteria (Anhydrous ammonia, nitrification inhibitors) (2) + Drainage tile prevents worms and microbes from flooding out (1) + Chicken litter (1) + Crop rotation reduces pests (1) + Fumigation reduces pests (1)	*"Well the little microbes are helping with your percolation, with your water coming through[…]They're keeping your soil alive. The soil is living and it's microbes that give it the life and the vitality. Without them you have dead soil. And that's why the cover crops come in handy, to keep things aerated. And those microbes live and die down there. They contribute your soil health and the organic matter." [MI3W113]* *"The less tillage you can do the more the earthworms like it as well. They don't like their homes disturbed." [MI3W111]*

2) HOW DO MICHIGAN FARMERS EVALUATE THE HEALTH OF THEIR SOIL?

2.1) MOST FARMERS USE SOIL TESTS, BUT FEW ASSESS MORE THAN THE CHEMICAL COMPONENTS OF SOIL

Most of the Michigan farmers we surveyed (77%) reported using tests to measure soil health at least sometimes. In interviews, most farmers described using traditional agronomic soil tests (e.g., pH, organic matter, and extractable nutrients), not tests that assess a broader array of 'soil health metrics'. Many of the interview participants (47%) reported using soil tests based on grid-sampling every one to three years to inform their fertility programs and, in some cases, their variable rate fertilizer applications (n = 31%). Despite their frequent use, there was still some confusion about the agronomic tests, such as why phosphorous levels do not always reflect fertilization recommendations, or what is available to the plant.

Only two farmers in the interviews used alternative soil health tests that also assess the biological properties of soil. One used the Haney test (Ward Laboratories, Kearney, Nebraska), which measures nutrient availability and biological activity through soil respiration, because it was part of a government funded program. The other partnered with a university to evaluate soil health metrics, including active carbon, water holding capacity and aggregate stability. Our results support other studies that also report low use of soil health tests by farmers (De Bruyn and Andrews, 2016; Mann et al., 2021; Wade et al., 2021).

Conversations with farmers also indicated that there is a need for improved translation of soil test results. For instance, the farmer that worked with a university to test his soil health shared that "[the soil health test] was great, because it was interesting, but I'm not sure what I learned, is the problem." [MI3W104] This quote emphasizes that, even if soil health tests become more accessible, that there is also a need to ensure that soil health tests move beyond "interesting". One way to do this, as discussed in a recently proposed framework for soil health assessments, may be to ensure that soil health indicators relate to management outcomes that are most interesting to farmers, such as yield and water quality (Wade et al., 2022). Doing this could also help farmers connect their understanding of soil health (mental models) with their management goals. For instance, changes in organic matter and soil biology, two of the top factors in farmers' soil health mental models, cannot be assessed easily, or even within a single season, which limit the extent to which they can inform management. Efforts to develop faster-responding indicators of organic matter (e.g., Permanganate Oxidizable Carbon) (Culman et al., 2012; Awale et al., 2017) or to determine how organic matter or soil biology impact yield (Oldfield et al., 2019)could help farmers relate their understanding of soil health to their crop productivity goals, and, in turn, increase their confidence in soil health practices.

2.2) FARMERS USE VISUAL INDICATORS TO ASSESS SOIL HEALTH DIFFERENCES ACROSS FIELDS, AND ATTRIBUTE DIFFERENCES IN SOIL TYPE TO SOIL HEALTH

In addition to using quantitative tests, we found that farmers define and evaluate soil health based on visual indicators of the soil and crops, as well as knowledge of field topography and soil types (Table 4.2). This is consistent with previous studies (Romig et al., 1995; Lobry De Bruyn and Abbey, 2003; Bagnall et al., 2020). Farmers often used visual differences in crops as indicators of soil properties, such as lower yields in the headlands as an indicator of soil compaction.

Farmers frequently mentioned differences in soil type when describing differences in soil health and yield across their farms. Heterogeneous soil types may be particularly prevalent in Michigan farms, as described by a farmer in our interviews: “you have so many different types of soil on one field […] from high to low to dark to light. It's just the way it is around here […] not like Illinois, where it's black from one end to the other.” [MI3W102]. Often these inherent differences in soil types were discussed in terms of soil health. Farmers described some soils in their fields as “just tough” or “like sandbox sand” and other soils, like loams, were described as “more alive”. Another farmer noticed that differences in the soil’s ability to hold water correlated with yield outcomes, but also questioned whether this reflects differences in soil health:

> “You can see the differences in productivity across the field. Is it soil health? Well, you know, a lot of it traces back to what areas hold water and which ones don't. You know, typically the areas that hold the water the best, are the areas that produce better.” [MI3W113]

This quote emphasizes how differences in soil types within a field can make it challenging for farmers to identify if yield differences emerge from variation in soil health, or from differences in yield potential, which may not be changeable within soil types.

Challenges with evaluating soil health across soil types also exists across larger boundaries, and, when used to compare yields, may unnecessarily make some farmers feel like “failures”, as described by a farmer that compared Michigan soils to the more naturally fertile soils in Iowa:

> “What’s good soil in one place, is maybe not considered good in another. The soil in our neck of the woods probably would not be considered very healthy to somebody from Iowa or Illinois […] If they raise raise 220 bushel an acre corn, that's considered a failure, [while] here [in Michigan], that's about the best we can do.” [MI3W101]

The same farmer went on to describe how the term “health”, itself, may be problematic when used to describe differences across soil types:

> “The use of the word ‘health’ kind of implies that some soil is not productive – is not healthy. And I'm not sure that’s it. It's a different kind of soil…it's not intended to be as productive, you know, it's just not going to be as productive. You know, that's not to say it's unhealthy.” [MI3W101]

This farmer is recognizing previously described challenges with soil health, namely that there are no universal benchmarks (Janzen et al., 2021). It might be useful to distinguish a definition of soil health that is based not on a number of absolute productivity but as a *potential* based on local comparisons to similar soil types. To this end, efforts should be made to create local databases that would allow farmers to compare their soil properties to other fields with similar soil types, historic land use and climates. Additionally, because previous studies suggest that farmers in different regions identify different indicators of soil health (Lobry De Bruyn and Abbey, 2003), locally-informed databases may also be more useful if they are reflective of the farmers’ preferred soil indicators, calling for studies similar to this in other states.

Table 4.2. Thematic coding for interview responses to “Do you think that soil health varies across your fields? And, if so, how can you tell?” 17 of the 19 farmers were asked this direction question; responses often included several indicators, so sample size varies.

Indicator of soil health	Indicator descriptions	Example Quotes
Crop visuals (9)	Yield; Leaf color (greenness, variegated purple leaves); Stress (dry leaves); Height	*“You're looking at your neighbor's and you look at yours and my crops might not be showing as much stress as theirs are. And I think okay, we're heading the right direction.” [MI3W114]* *“Well, ultimately, the yield usually will bear that out. You can tell sometimes there's a discoloration. But usually, if there's a discoloration if there's going to be a yield indication at the end of it.” [MI3W115]*
Soil visuals (9)	Soil color; Standing water; Compaction; Ability to work (e.g. mellow)	*“We've got some [fields] […] that's kind of a black, sandy soil sand underneath. And it's very nice to work. It's very mellow.” [MI3W117]* *“Yeah, and I think probably a lot of the visual is more so compaction and the ability for water to percolate or, or pool, you know, in a low spot are a compacted spot that holds water, that the corn is short, it's yellow, it just is not as thrifty.” [MI3W110]*
Field position (6)	High vs. low areas; Headlands; Buried stones in the fields; Irrigated soils are healthier; Proximity to fence rows and trees	*“Our end rows, those are going to be gravelly and hard and compacted. So those always yield less. Once you get to the middle of the field. It's gonna be better.” [MI3W102]* *“Yeah, the non-irrigated ground will be more of a yellowish sand than what the irrigated is. Like I said, I've got the irrigated down to where it's almost dark brown or almost black at the top.” [MI3W116]* *“When I work […] the soil around the fence rows […] near the trees, the soil is always…it looks better.” [MI3W101]*
Soil type (6)	Sandy, loamy, mucky; Ability to hold water	*“Well, you know, a lot of it traces back to what areas hold water and which ones don't […] typically the areas that hold the water the best, are the areas that produce better” [MI3W113]* *“Just by soil type…our farms range anywhere from sand, which is very light soil to...and you go down into lower areas too muck. So the soil health in the lower areas is much better than up on the sand hills.” [MI3W105]* *“You get in some of the sandy soils and just like, sandbox sand. That you grab some of the lower, like, lower loamy ground and so it's, it's like it's more alive.” [MI3W111]*

3) HOW DOES SOIL HEALTH INFORM FARMERS' MANAGEMENT DECISIONS?

3.1) FARMERS' TOP MANAGEMENT CONCERNS AND HOW THEY RELATE TO SOIL HEALTH

Many farmers' mental models of crop productivity included soil properties (12 out of 35 topics), but crop characteristics (n = 3), management practices (n = 9), and external factors (n = 10), like weather and markets also were well-represented (Figure 4.5). The topics "weather", primarily described as precipitation (both too little and too much), and "diseases and pests" were the most frequently mentioned factors to impact crop productivity, potentially suggesting that these are also farmers' greatest concerns for management. Data from survey and interviews suggest that farmers see a role for soil health in buffering crop productivity from water stress. In interviews, farmers frequently discussed how organic matter and soil biology play a role in water management (discussed previously, Table 4.1). Similarly, in the survey nearly all farmers (93%) reported that they believe healthy soils can increase drought resilience (Figure 4.2), as supported by recent literature (Hewitt et al., 2021; Renwick et al., 2021)

"Diseases and pests" was the second most common topic in the farmers' crop productivity mental models (n = 12/20, Figure 4.5). This topic was broad, and included a variety of pests, including weeds, fungi, and insects. In the interviews few farmers described a relationship between soils and pest pressure, consistent with discussions during a recent soil health workshop (personal communication, June 29, 2022). Highlighting the opportunity for soil health to reduce pest pressure (Janvier et al., 2007; Larkin, 2015) could be an additional strategy for soil health educators to align efforts with farmers existing management priorities.

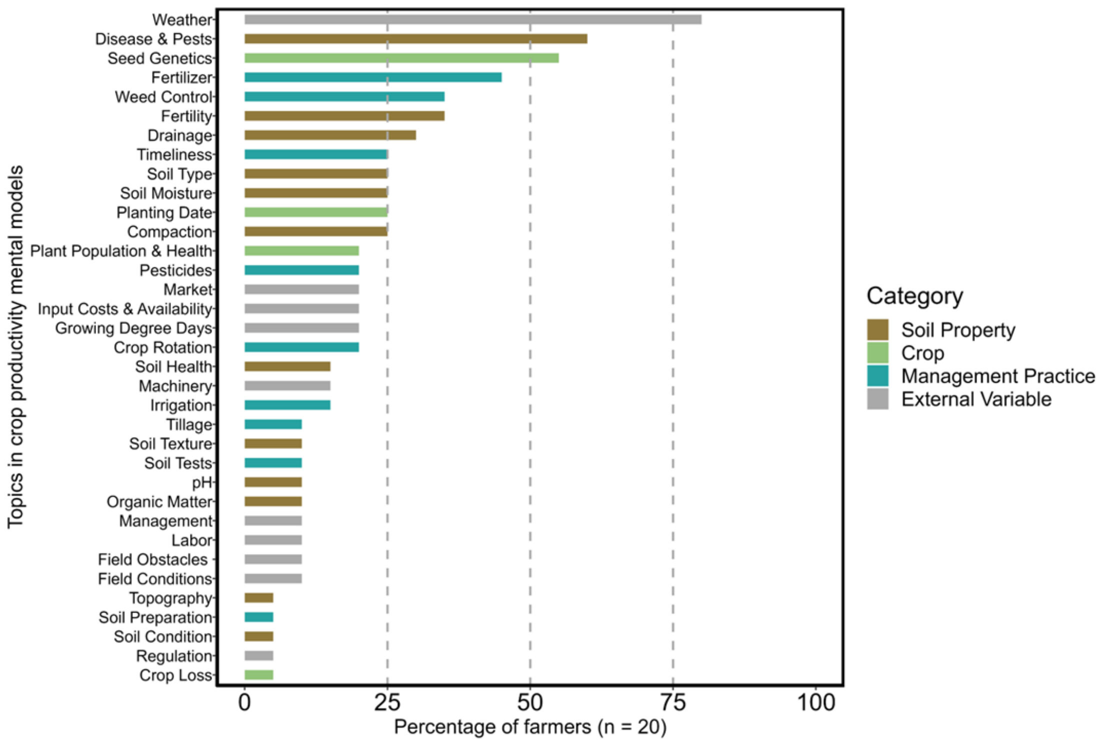

Figure 4.5. Topics included in Michigan farmers' crop productivity mental models, each topic consisted of several terms aggregated (Table 4.S2). Colors reflect the category of the term, as defined by the lead researcher. Gray-dashed lines indicate 25%, 50%, and 75% of farmers. The term "drainage" sometimes referred to the natural ability of the soil to hold water (soil property), but also tile drainage (management practice).

3.2) FARMERS USE A VARIETY OF PRACTICES TO TARGET SOIL HEALTH, BUT CLAIM THERE IS MUCH MORE TO BE DONE

We investigated how farmers' understanding of soil health influences management in two ways: first, we evaluated if the topics included in the soil health mental models differed for farmers that use or do not use cover crops. We found that farmers that used cover crops included more topics, especially those other than organic matter and compaction, suggesting that they may have broader understanding of soil health (Figure 4.S3). Second, we examined differences in farmers' perceived knowledge and use of cover crops, using survey data. We found that farmers' use of cover crops only weakly correlated with their perceived knowledge of soil health ($r = 0.10$, $p = 0.056$), but more strongly correlated with their perceived knowledge of using cover crops ($r = 0.46$, $p < 0.001$). These data suggest that farmers' management decisions are more likely to be informed by knowledge of specific practices, rather than by the more general topic of soil health. Other studies also show that farmers' knowledge of and experience with cover crops correlates with their adoption (Singer et al., 2007; Arbuckle and Ferrell, 2012), but that knowledge is not the only barrier to adoption (Bergtold et al., 2012; Reimer et al., 2012; Burnett et al., 2018). We also identified other barriers; several farmers discussed the important role of cover crops for soil health but were not using the practice, in part because they were nearing the end of their farming careers.[8]

To further study drivers of adoption, we tested whether farmers that are "taking steps to improve the health of soils they farm" are also using soil health practices. Most farmers (86%) say they are taking steps to improve the health of soils (Figure 4.3), and a similarly high percentage (78%) are using either no-till or cover crops on at least some fields in their operation.[9] This suggests that cover crops and no-till are practices that farmers use to manage for soil health, consistent with what was shared in the interviews (Table 4.1). A smaller percent of farmers (11%) said they have taken steps to improve soil health but do not use cover crops or no-till. In the interviews, farmers shared that try to improve soil through a wide variety of practices other than those listed in our survey, such as manure, biological products and soil amendments, or even bio-solids (Table 4.1). It is also possible that farmers' self-reported "steps for improving soil health" may include practices that are not often attributed to building soil health, like deeper tillage practices. Indeed, while most farmers described how no-till helps build soil health, one farmer said chisel plowing puts "stuff down in the ground" [MI3W107] which is good for building organic matter (Table 4.1). This farmer's perception may exemplify a previously observed pattern whereby farmers' use their identify as stewards of the land to justify that their practices are good for the soil (Roesch-McNally et al., 2018; Irvine et al., 2023). Consistent with most farmers' efforts to improve soil health, we found that farmers view caring for the soil as part of the job, and that there are always things that can be done:

> "I'm trying to do everything I can to make this ground produce what it can for me and my kids and my grandkids. Soil health is obviously a big part of that. So I try to actively do what I can to

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[8] Even farmers that did not use cover crops included them as an important practice in their soil health mental models (n = 2/8, Figure 4.4), and multiple farmers that discussed the role of cover crops for building organic matter also did not use the practice (n = 5/13, Table 4.1). One farmer that recognized the benefits of cover crops shared that he is unlikely to use them because he is nearing the end of his farming career: "[Other farmers] claim they're getting more organic matter in the soil by doing that [planting cover crops]. It kind of makes sense, but at this point in our careers, we're going to wait for the next team to take over and do whatever they think is best." [MI3W103]

[9] 47% of farmers use either no-till or cover crops on at least some fields and 31% use both practices on at least some fields

> maintain or improve where I can. But to say that I've exhausted every effort to improve just soil health, I haven't gotten there yet.” [MI3W104]

Farmers may not have a specific plan for improving soil health, but they find it to be an unquestionable part of their lives, comparing it to living a healthy life, or as their personal mission statement:

> “Yeah, I think [I try to build soil health]. It's, you know, do you have a real plan to keep you healthy, or do you just kinda live right. You know, I don't know if I have a real plan to keep me healthy, but I don't smoke. I don't drink much. And I you know, I exercise. It's kind of the same way with the farm things. I try not to do anything that I know is, is bad. I don't know, I don't have a real scientific plan.” [MI3W117]

> “So it's like, some people have to have a mission statement to get out of bed. I don't need one of those. I know what my mission is. So that is just how I operate. So it would be nice to have a soil management plan, like a PDF somewhere on the wall. But that hasn't become priority one yet.” [MI3W104]

These quotes support previous literature showing that soil health is an important part of farmers' stewardship ethic (Roesch-McNally et al., 2018; Bagnall et al., 2020). Given this, it is not surprising that Michigan farmers are taking steps to improve soil health on their farms. The bigger question, then, is: are their management efforts working? To get at this question, future studies should focus on mixed-method approaches, such as first using qualitative research to assess the breadth of soil health practices and evaluation techniques that farmers are using, followed by quantitative methods to assess the effectiveness of the practices over time, as well as what soil health indicators respond most quickly to management (e.g. O'Neill et al., 2021).

SUMMARY AND CONCLUSIONS

Using survey, mental modeling and interview methods, we investigated how Michigan farmers understand, evaluate, and manage for soil health. There were three primary conclusions from this study:

1) Michigan farmers believe in the benefits of soil health and have a complex, systems-level understanding of it, but that it can be challenging to apply this knowledge to management.

Nearly all farmers reported that they believe healthy soils have the potential to increase yields and drought resilience. Using mental models, we found that organic matter and soil biology were central to farmers' soil health mental models, and that they identified many mechanisms and management practices that affect these properties. However, these properties were less commonly used to guide management, likely because they cannot be easily measured or related to outcomes that are most of interest to farmers.

2) Farmers primarily assess soil health with agronomic nutrient tests and visual indicators, such as yield and leaf coloration, but soil type can make yield a misleading indicator.

In Michigan, soil types are very heterogenous, and because differences in yield can be attributed to soil type, it can be a problematic indicator of soil health. Regionally-specific benchmarks could help farmers differentiate the effects of soil health and soil type on crop yields.

3) Many Michigan farmers have adopted no-till and cover crops to improve soil health, and express that soil health is a top priority, though it may not always be reflected in their management concerns.

In the survey, most farmer said they have taken steps to improve soil health, and this was generally supported by their use of cover crops and no-till. Still, soil health and conservation practices were not frequently included in farmers' crop productivity mental models even though, in interviews, farmers described caring for the soil as part of their personal mission statement. In mental models, weather and pest pressure were identified as top management concerns. Because soil health has the potential to mitigate drought stress and pest pressure, focusing on these relationships could help farmers relate soil health to their management priorities.

Overall, we found that Michigan farmers have deep knowledge and appreciation for soil health and that there are many research and outreach opportunities that could help farmers more intentionally fit soil health into their management decisions (Box 1).

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APPENDIX A: CHAPTER ONE SUPPLEMENTAL METHODS AND RESULTS

ROOT MORPHOLOGY WITH GIA ROOTS

Root morphologies were quantified using a skeletonization algorithm applied to images of roots produced using an Epson perfection V600 scanner. After scanning roots at 1200 DPI, the images were edited manually with Adobe Photoshop Elements 16 to remove image artifacts and then resized to 300 DPI. GiA Roots (Galkovskyi et al. 2012) software algorithm masked roots against the image background using the adaptive image thresholding feature and a set of manually evaluated parameters (mean shift, minimum component size, block size). After masking and identifying roots in the image, GiA Roots trimmed imaged roots down to a diameter of a single pixel and measured root length by dividing total skeleton pixels by a known conversion factor established by a ruler image.

UNIVARIATE DATA ANALYSIS

Prior to all data analysis, we assured that all univariate data met assumptions of normality; transformations for normality included: predicted dry root biomass (square-root), root length density (square-root), GiA Roots specific root length (log), soil moisture content (log), and soil ammonium and nitrate (log +1), and root-bacterial evenness (squared). Soil fungal Shannon diversity and evenness indices were not able to be normalized, so we used non-parametric Kruskal-Wallis and Wilcox-tests with cultivar or ecotype as fixed effects (no block effect). However, although the data was non-normal, we confirmed that fungal Shannon diversity had the same results with a the mixed-effects model with a block factor included. Two extreme outliers that were three times the interquartile range were removed from soil moisture data and these two datapoints were also removed from soil nitrate and ammonium data, as soil moisture content data was used to normalize nitrogen values per unit of dry soil. Several datapoints for microbial biomass carbon were negative, likely because carbon values were lower than the instrument's standard error. These negative values were omitted from the analysis.

MVABUND ANALYSIS OF TAXA GROUPING AND CORRELATIONS WITH ROOT TRAITS

The 'manyglm' function in the MVAbund R-package was used to identify bacterial and fungal taxa that had significantly different relative abundance among cultivars, ecotypes, or plant compartments (Wang et al. 2012). Cultivar, ecotype, or soil type (root or soil) were treated as fixed effects in a "negative-binomial"-fit model. Block could not be included as a random factor due to unequal replication across blocks (because of samples removed for poor-sequence coverage). Taxa that significantly differed among groups ($p < 0.05$) were then analyzed with ANOVA tests (FDR adjustment to correct for multiple testing, $\alpha = 0.05$) with either cultivar or ecotype as a fixed effect. Soil moisture content was included as a covariate to account for variation across sample dates. Relative abundance data was log-transformed when it did not meet assumptions of normality. Further, we used the manyglm model to identify if the abundance of any fungal or bacterial groups (classes or orders) or individual OTUs (OTUs present in at least 80% of the samples) correlated with root length or diameter. Continuous root length and average root diameter data were fit with the negative-binomial manyglm model. Significant relationships between root traits and microbial orders or OTUs found with MVAbund were confirmed with a linear regression analysis.

NITROGEN-FIXATION CAPACITY ESTIMATES

The mean relative abundances of Burkholderiales and Rhizobiales were calculated using the rarefied soil (12 cultivars) and combined root and soil (4 cultivars) bacterial datasets, then analyzed with the non-parametric Kruskal-Wallis tests (R Core Team, 2018) with either cultivar, ecotype, or sample type as main effects. We also approximated the N-fixation capacity of the soil (12 cultivars) and root (4 cultivars) bacterial communities using PICRUSt (Langille et al. 2013). PICRUSt infers function based on phylogenetic relatedness to a database of reference genomes, so is only an approximation due to the tenuous and highly variable relationship between 16S rRNA sequence and function. We first calculated nearest sequenced taxon index (NSTI) scores, which provides a measure of phylogenetical distance between each OTU and the referenced metagenome and describes the confidence in functional assignment (Langille et al. 2013). We normalized all OTUs by their predicted 16S rRNA gene copy number, which provides a pseudo-abundance estimate for each OTU and then used 'metagenome_predictions' to obtain OTU-specific gene counts for N-fixation using the following KEGG pathway orthologs: K02588, K02586, K02591, K00531. We calculated each samples' predicted proportion of N-fixation genes by dividing the number of OTUs with at least one predicted N-fixation pathway for each sample by the normalized abundance of OTUS (e.g., the total 16S-gene normalized OTU counts).

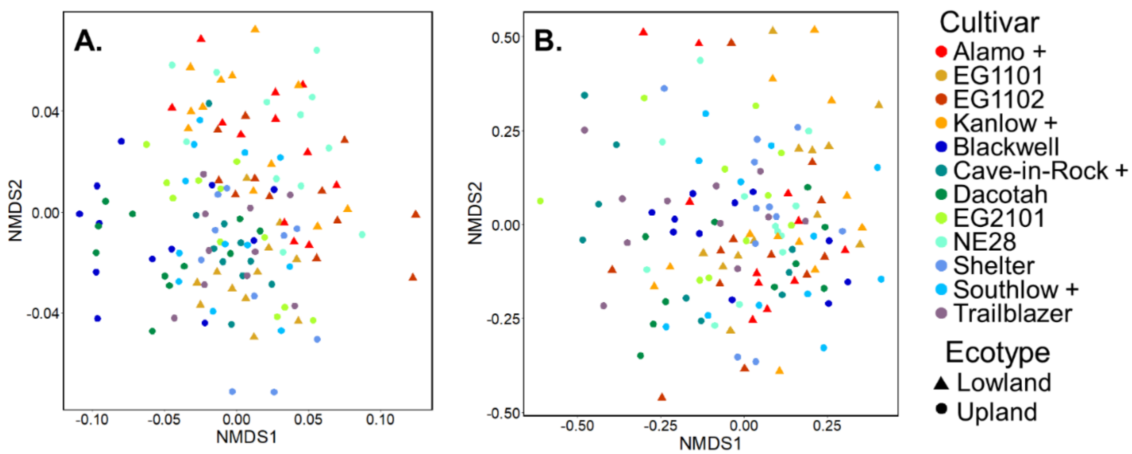


Figure 1.S1. NMDS ordination of A) soil bacterial community (Weighted Unifrac, stress = 0.18) and B) soil fungal community (Bray-Curtis, stress = 0.26). Each point is a replicate soil core; final replicate number for each cultivar after removing poor sequence coverage samples in Table S2. Warm colors and triangles represent lowland ecotypes (n = 4), cool colors and circles represent upland ecotypes (n = 8). '+' denotes subset of cultivars analyzed for root-associated bacterial communities.

Table 1.S1. Bioinformatics filtering details for bacterial (16S) and fungal (ITS) samples.

	Bacterial root and soil	Bacterial soil	Bacterial soil (PICRUSt analyses)	Fungal Soil
Reference Database	Silva (v.123)	Silva (v.123)	Greengenes (v.13.8)	Unite (v.7.2)
Total Read #	3,323,839	2,294,871	2,031,361	2,202,804
Total OTU # (97% similarity threshold)	20,972	20,278	11,931	4,736
% non-bacterial or fungal reads	19.07%	0.79%	0.72%	0%
# samples after removing poor-sequence coverage	182 (removed 10 samples)	138 (removed 5 samples)	138 (removed 6 samples)	135 (removed 9 samples)
Post-filtering Read #	2,680,275	2,267,356	2,009,262	2,196,278
Post-filtering OTU#	18,535	17,878	8,878	4,639
Rarefaction Read # cut-off	2,026	4,694	4,117	4,153
Post-Rarefaction Read #	368,732	647,772	568,146	560,655
Post-Rarefaction OTU#	12,197	14,590	7,905	4,064
Protest results (comparing rarefaction and Deseq2 VST normalization)	$p < 0.001$, r = 0.41	$p < 0.001$, r = 0.91	NA	$p < 0.001$, r = 0.82

Table 1.S2. Fungal and bacterial sequencing final replicate number (out of 12 replicates; 4 blocks with 3 replicate soil cores) after removing samples with poor sequence coverage and samples with extreme outliers for soil moisture content covariate (n = 1 from EG1101 and n = 1 from Blackwell). 'NA' denotes not applicable; only 4 cultivars analyzed for root bacterial community.

	Alamo	EG1101	EG1102	Kanlow	Blackwell	Cave-in-Rock	Dacotah	EG2101	NE28	Shelter	Southlow	Trailblazer
Soil Bacterial Community	12	12	12	12	12	10	11	11	12	10	12	10
Combined Soil, Root bacterial community	12, 9	NA	NA	12, 11	NA	10, 10	NA	NA	NA	NA	12, 12	NA
Fungal Bacterial Community	11	12	12	12	11	9	10	10	12	11	12	11

Table 1.S3. Percent variability (PERMANOVA R^2) in microbial community composition explained by cultivar or ecotype. Cultivar or ecotype treated as main effects with sampling date as a covariate and a nested block term. Significance values: ns $p > 0.05$, *$p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$. '()' signifies nested factors and '*' signifies the interaction between factors.

Factor	Soil fungi (12 cultivars)	Soil bacteria (12 cultivars)
Cultivar Effect	%R^2 (p)	%R^2 (p)
Cultivar	ns	13.03**
Block (Cultivar)	34.23***	33.77***
Sampling Date	1.56**	9.13***
Ecotype Effect		
Ecotype	ns	ns
Plot (Ecotype)	43.31***	45.79***
Sampling Date	1.56***	9.13***

Table 1.S4. Percent variability (PERMANOVA R2) in bacterial community composition explained by habitat (soil or root) and cultivar. Cultivar and habitat treated as main effects with sampling date as a covariate and a nested block term. Cultivar-effect for a subset of soil and root communities also presented; NA indicates not applicable for the model. Significance values: ns $p > 0.05$, *$p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$. '()' signifies nested factors and '*' signifies the interaction between factors.

Factor	Soil & root bacteria (4 cultivars)	Soil bacteria (4 cultivars)	Root bacteria (4 cultivars)
Habitat Effect	%R^2 (p)	%R^2 (p)	%R^2 (p)
Cultivar	ns	ns	ns
Block (Cultivar)	6.42 *	32.9 ***	ns
Habitat	58.72 ***	NA	NA
Cultivar*habitat	ns	NA	NA
Habitat*Block(Cultivar)	6.71 *	NA	NA
Sampling Date	1.67 **	10.34 ***	ns

Table 1.S5. Root trait differences among switchgrass cultivars and ecotypes. ANOVA results with fixed cultivar or ecotype term, nested block term, and soil moisture content as a covariate when it improved model fit (based on Akaike information criteria). F-statistic and significance values: ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, 'NA' denotes not-applicable for the model.

	Dry Root Mass (g)	Average Root Diameter (cm)	Root Network Volume (cm3)	Root Network Length (cm)	Volume-weighted SRL (cm/cm3)	Mass-weighted SRL (cm/g)
Cultivar Effect						
Cultivar	1.61 (ns)	4.43***	1.99 (ns)	1.21 (ns)	3.61**	1.62 (ns)
Soil Moisture	NA	NA	NA	0.73 (ns)	NA	NA
Ecotype Effect						
Ecotype	2.47 (ns)	0.001(ns)	1.49 (ns)	3.32 (ns)	0.288 (ns)	2.43 (ns)
Soil Moisture	NA	NA	NA	0.99 (ns)	NA	NA

Table 1.S6. Bacterial alpha diversity among root and soil habitats. ANOVA results with habitat and cultivar (n = 4) as fixed terms, a nested block term, and soil moisture content as a covariate when it improved model fit (lower Akaike information evaluation). Wilcox test with compartment as a fixed effect was used for non-parametric Pielou's evenness. F-statistic and significance values: ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, 'NA' denotes not-applicable for the model.

	Richness	Shannon Diversity	Pileou's Evenness
Habitat	3509.8 ***	1178.1 ***	W = 1 ***
Cultivar	0.16 (ns)	1.73 (ns)	NA
Habitat * Variety	1.06 (ns)	1.74 (ns)	NA
Soil Moisture	2.15 (ns)	NA	NA
Habitat Means (Soil, Root)	889, 171	38.1, 14.2	0.91, 0.73

Table 1.S7. Alpha diversity statistics for soil bacterial and fungal communities. ANOVA results with either fixed cultivar or ecotype term, nested block term, and soil moisture content as a covariate when it improved model fit (lower Akaike information evaluation). Fungal Shannon diversity was analyzed with non-parametric Kruskal-Wallis and Wilcox Tests. F-statistic and significance values: ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, 'NA' denotes not-applicable for the model.

Community	Bacterial Soil Community (n = 12 cultivars)			Fungal soil community (n = 12 cultivars)		
Diversity Metric	Richness	Shannon Diversity	Pielou's Evenness	Richness	Shannon Diversity	Pielou's Evenness
Cultivar Effect						
Cultivar	2.17*	4.4***	4.71***	0.63 (ns)	X2 = 7.22 (ns)	X2 = 8.98 (ns)
Soil Moisture	0.65 (ns)	NA	NA	1.40 (ns)	NA	NA
Ecotype Effect						
Ecotype	2.18*	6.15*	5.41**	0.04 (ns)	W = 2177 (ns)	W = 2109 (ns)
Soil Moisture	0.65 (ns)	NA	NA	0.10 (ns)	NA	NA
Ecotype Means (Upland, Lowland)	1460,1416	6.49, 6.41	0.89,0.88	6.70, 9.26	4.47,4.42	0.76,0.75

Table 1.S8. Alpha diversity statistics without Dacotah cultivar for soil bacterial community (n = 11). ANOVA results with either fixed cultivar or ecotype term, nested block term, and soil moisture content as a covariate when it improved model fit (lower Akaike information evaluation). Fungal Shannon diversity was analyzed with non-parametric Kruskal-Wallis and Wilcox Tests. F-statistic and significance values: ns $p > 0.05$ *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, 'NA' denotes not-applicable for the model.

Diversity Metric	Richness	Shannon Diversity	Pielou's Evenness
Cultivar Effect			
Cultivar	2.08 (ns)	2.14 (ns)	2.06 (ns)
Soil moisture	0.27 (ns)	NA	NA
Ecotype Effect			
Ecotype	2.47 (ns)	3.72 (ns)	2.96 (ns)
Soil moisture	4.18 *	NA	NA
Ecotype means (Upland, Lowland)	1448,1416	6.47,6.41	0.89,0.88

Table 1.S9. Pairwise *p*-values (FDR adjusted) for soil bacterial community composition among lowland (L) and upland (U) cultivars. Model included cultivar as a fixed effect with a nested block term and soil moisture content as a covariate. Shading represents *p*-value < 0.1 Final column denotes how many of the 11 comparisons for each cultivar were significant at *p* < 0.10.

	Alamo	EG1101	EG1102	Kanlow	Blackwell	Cave-in-Rock	Dacotah	EG2101	NE28	Shelter	Southlow	Trailblazer	# *p* < 0.1
Alamo (L)													7
EG1101 (L)	0.09												4
EG1102 (L)	0.61	0.09											6
Kanlow (L)	0.92	0.09	0.32										4
Blackwell (U)	0.09	0.13	0.09	0.17									3
Cave-in-Rock (U)	0.09	0.25	0.09	0.09	0.22								4
Dacotah (U)	0.09	0.15	0.09	0.09	0.82	0.19							4
EG2101 (U)	0.09	0.18	0.09	0.13	0.19	0.63	0.19						3
NE28 (U)	0.92	0.09	0.57	0.79	0.09	0.09	0.09	0.09					6
Shelter (U)	0.09	0.93	0.15	0.09	0.15	0.44	0.18	0.61	0.09				3
Southlow (U)	0.16	0.57	0.09	0.17	0.21	0.9	0.21	0.92	0.13	0.64			1
Trailblazer (U)	0.09	0.52	0.15	0.13	0.19	0.57	0.19	0.57	0.12	0.64	0.75		1

Table 1.S10. Percent variability (PERMANOVA R2) for cultivar effect on soil bacterial and fungal communities within sampling dates. Significance values: ns $p > 0.05$, *$p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$; R^2 = Factor SS/Total SS.

Sampling Date	June 28th	July 13th	July 20th	July 27th
Soil bacterial community	%R^2 (*p*)	%R^2 (*p*)	%R^2 (*p*)	%R^2 (*p*)
Cultivar	ns	ns	0.16**	ns
Block(Cultivar)	48***	36**	33***	41***
Soil fungal community	R^2 (*p*)	R^2 (*p*)	R^2 (*p*)	R^2 (*p*)
Cultivar	ns	ns	ns	ns
Block(Cultivar)	37***	36***	33**	36***
# cultivars sampled	2	3	4	3

APPENDIX B: CHAPTER TWO SUPPLEMENTAL METHODS AND RESULTS

SEEDLING PREPARATION

We sterilized all species' seeds by submerging them for 15 minutes in 5% NaHCl, followed by three five-minute rinses with sterile water. To improve germination, *P.virgatum* seeds were imbibed in for 5 days at 4 °C prior to sterilization. The sterilized seeds were planted into germination flats that contained a sterile base soil (autoclaved sand, vermiculite and sphagnum peatmoss) with a light layer of field soil inocula to provide the plants with an initial microbial community. The soil inocula was the same used in the greenhouse experiment (described in main text). The seedlings grew for five weeks and were fertilized with half-strength Hoaglands' micronutrient solution throughout this period (total equivalent of 79 kg N ha^{-1} $flat^{-1}$).

MICROBIAL BIOMASS AND NITROGEN ANALYSES

We used unfumigated potassium sulfate extractions to determine the total dissolved organic carbon (DOC) and total extractable soil nitrogen (TN) in each pot (Shimadzu TOC-VCPH). Microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) were calculated by subtracting total C or N of the unfumigated samples from the fumigated sample and dividing the difference by previously calibrated extraction efficiencies (K_{EC}: 0.45; K_{EN}: 0.54) (Joergensen, 1996; Joergensen and Mueller, 1996). Unfumigated potassium sulfate extracts were also used to determine the amount of soil inorganic nitrate (NO_3^-) with a colorimetric 96-well plate assay using nitrate reductase (adapted from (Sinsabaugh et al., 2000). Absorbance values were read on a BioTek plate reader and negative values for DOC, TN, NO_3^-, NH_4^+ were converted to zeros for all statistical analyses.

ROOT EXUDATE ANALYSIS: LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (LC-MS)

For LC-MS, each exudate sample (100 µL of extract) was added into a high-resolution LTQ Orbitrap Velos mass spectrometer (HRMS) with a heated electrospray ionization (HESI) source (Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled to a Vanquish ultra-high pressure liquid chromatography (UHPLC) system (Thermo Fisher Scientific). Chromatography was performed on a C18 Hypersil gold reversed-phase column (150 × 2.1 mm, 3µ particle size; Thermo Scientific) operating at 30 °C. Mobile phases consisted in 0.1% formic acid in water (A) and acetonitrile/0.1% formic acid in water (90:10; B), with a consistent ejection volume (5 µL) and flow rate (0.3 mL $minute^{-1}$). For each sample, the elution gradient initiated at 90% A (10% B) gradient ramped to 10% A (90% B) after five minutes, and remained for the 15 minutes; these conditions were maintained for two minutes before the initial proportions (90% A; 10% B) were linearly recovered. The column was washed and stabilized for 11 minutes at the initial conditions before the injection of the next sample. The HRMS operated in

Fourier Transform Mass Spectrometry (FTMS) tandem MS, obtaining MS1 and MS2, and full-scan mode at a resolution of 60,000 acquiring masses between 50-1000 m/z. Experimental blanks (methanol:water, 80:20) were run every ~10 samples for noise level determination.

The chromatograms (HRMS RAW files) were baseline corrected, deconvoluted, and aligned in MZmine 2.37 (MZ tolerance of 6ppm & RT error of 0.3 min) (Pluskal et al., 2010), followed by a second-level of metabolic feature annotation (Sumner et al., 2007) using the exact mass (MS1) and retention time (RT) of over 600 standard compounds included in EMSL's LC-MS library.

ROOT EXUDATE ANALYSIS: GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS)

For GC-MS analyses, each exudate sample (200 µL of extract) was dried and posteriorly derivatized. The dried extracted metabolites were first derivatized to trimethylsilyl ester form (Kim et al., 2005) by adding 20 µl of methoxyamine in pyridine solution (30 mg/mL) to each sample and incubating them in a Thermomixer at 1,200 rpm for 90 min at 37 °C. After the first incubation, each vial received 80 µl of MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) and incubated at 1,200 rpm for 30 min at 37 °C to derivatize amine, carboxyl and hydroxyl groups. Finally, vials were vortexed for 10 seconds and centrifuged for 1 min at 8,000 rpm. Supernatants were thus transferred into a new set of glass vials.

We analyzed the derivatized samples with an Agilent GC 7890A coupled to a MSD 5975C mass spectrometer (Agilent Technologies, Santa Clara, CA) and a HP-5MS column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies). We maintained a consistent injection volume (10 µL) and the injection port temperature was maintained at 250 °C. The column oven was held for one minute at 60 °C before increasing the temperature to 325 °C at a rate of 10 °C/min and holding for an additional ten minutes. A mixture of fatty acid methyl esters (FAMEs; C8-C28) were analyzed at the beginning of the sequence to calculate the retention indices of the detected compounds.

The GC-MS chromatograms (RAW files) were deconvoluted, aligned and metabolites were identified with Metabolite Detector 2.5 (Hiller et al., 2009). Initial metabolite identification was performed by matching MS spectra and RT, calculated through the analyzed FAMEs mixture, to an updated in-house version of FiehnLib (Kind et al., 2009) which contains over 850 metabolites with validated spectra and RIs. Assigned metabolites were subsequently validated by matching fragmented spectra from the NIST14 GC-MS library.

ROOT EXUDATE ANALYSIS: DATA PROCESSING

All putative compound assignments were filtered following a series of conditions based on differences between the matched metabolite and the RT and m/z values of real standards. If features met the following criteria, they were identified as 'unknown':

- Features assigned with m/z error >= 6 ppm after mass calibration
- Features eluting between 2 and 10 minutes with RT error > 0.3 min respect to the elution time of standards
- Features eluting before 2 minutes with RT error > 0.25 minutes
- Late eluting features (> 10 min) with RT error between 0.3 - 0.5 min and m/z error >= 4

We combined the LC-MS and GC-MS databases by checking for duplicate feature name assignments between the two databases. If features were identified to have the same feature name, then we summed the peak areas from either dataset.

With the merged LC- and GC-MS dataset, we filtered out biologically relevant zeros from instrument detection issues by determining the proportion of samples in each treatment (n = 5 per treatment) that detected each metabolic feature. If 60% of the samples (3/5) detected the feature, then the samples with zero were given the average intensity of the other samples in the same treatment. However, if more than 60% of the samples were zeros, then the other samples also were made to zero. Because the initial exudate volume varied among samples and the amount of total carbon in the exudates did not significantly differ among treatments (total organic carbon: $F_{1,18} = 1.12$, $p = 0.379$) we normalized the remaining metabolic features by the relative abundance of metabolic features within each sample (peak intensity of each compound divided by the total ion chromatogram intensity).

DNA SEQUENCING AND BIOINFORMATICS

All DNA extraction kit-suggested protocols were followed, except for an added 10-minute cell lysis step at 65 °C before the bead-beating step. The purity and quantity of the extracted DNA was examined using nanodrop (Nanodrop 2000) and PicoGreen Fluoremetry (Quant-iT PicoGreen), respectively. We targeted the bacterial V4 region of the 16S rRNA gene (primers 515f/806r) and MiSeq Illumina (V2) paired-end sequencing was conducted by the Research Technology Support Facility Genomics Core at Michigan State University, East Lansing, Michigan. Briefly, the 16S reads were assembled, and quality filtered (discarded reads with quality filter maxEE < 1.0 and base pairs < 250) using Usearch (version 10.0.240) (Edgar, 2010). Sequences were dereplicated, clustered, chimera checked, filtered de novo, and clustered into unique operational taxonomic unites (OTUs) based on 97% identity using the default settings with Usearch UPARSE function (Edgar, 2013). Representative sequences were aligned and classified using the Silva (version 123) bacterial database at 80% confidence (Quast et al., 2012). We removed non-bacterial sequences (e.g. archaea, mitochondria, chloroplasts, consisting of 2.3% of sequences) and singleton OTUs, resulting in 11,690 bacterial OTUs (2,148,296 sequences).

We rarefied all samples to 16,224 reads (losing 495 OTUS during rarefaction) to control for community differences due to variation in sampling effort. We confirmed that our results were robust to normalization strategies by comparing multiple normalization methods (rarefied dataset, untransformed, compositional data, and data normalizehortd with Deseq2's Variance Stabilizing Transformation) with Protest (p <0.001, r > 0.97). We removed any taxa that were not present at least 10 times across either the greenhouse or incubation datasets, which resulted in 6,221 taxa for the greenhouse dataset (5,713 in the focal treatments alone) and 4,234 taxa in the soil incubation dataset.

DATA ANALYSIS

For all statistical models, no blocking structure was included, as there were only five replicates and no blocking design in the treatments. Neighbor-induced variation in the bacterial communities and root exudates were determined using PERMANOVAs with the Vegan R package Adonis2 function. We controlled for the effect of neighbor plant competition by including focal plant biomass as a covariate in the model. The term 'by' was set to 'margin' in the model so that the order of the fixed variable and covariate did not affect the results.

Indicator genera were determined for both the focal plant treatments and soil incubation treatments by merging samples by Genus with the 'tax_glom' function in the Phyloseq R package followed by indicator species analyses with the 'multiplatt' function in the indicspecies R package, and an additional FDR p-adjustment using the 'p.adjust' function (Caceres and Legendre, 2009). For the soil incubations, we used differential abundance analysis with the ANCOM R package to determine which bacterial taxa were enriched in the high malic acid soil treatment, and then evaluated if the abundance of these genera also differed for the greenhouse experiment.

To further partition variation in the focal plant root exudates, we used sparse Partial Least Squares Discriminant Analysis (sPLS-DA) to determine which of the identified metabolites contributed to the greatest variation in treatments using the MixOmics R package (Gonzalez-Dugo et al., 2012; Rohart et al., 2017). The sPLS-DA models were tuned and optimized using the 'tune.splsda' and 'perf' functions: model selection determined that four components with 50, 40, 20, and 40 features for each compartment were needed. The maximum distance error rate for four components was 0.12, and the error rates were highest for the no neighbor control and *A. gerardii* neighbor treatment.

Distance-based Redundancy Analysis (dbRDA) was used to evaluate how the top ten most abundant identified exudates, as well as the focal plant soil and plant characteristics, influenced bacterial community structure. Before the analysis, the metadata were scaled by their Z-score and we identified the most parsimonious model with the 'ordistep' function (forward selection). Only two metabolites, malic acid and stearic acid, and one plant characteristic, focal plant shoot CN, were identified as significant drivers of the bacterial community.

We looked for correlations between bacterial genera and the identified metabolites using the CCREPE R package. This new package improves upon traditional correlation techniques, such as Pearson and Spearman, by providing permutationally-based p-values and q-values (equivalent to FDR-adjusted *p*-values), and a novel similarity measurement of co-occurrence (nc.score) that is more suitable for compositional data. The nc.score is analogous to values typical of standard correlation measurements; we defined significant and strong correlations as those with $q < 0.05$ and $nc > 0.40$.

Table 2.S1. Effect of neighbor treatment on focal plant bacterial community and root exudates, as well as differences among monoculture treatments in greenhouse experiment. ANOVA and PERMANOVA results show main effect of neighbor treatment with focal plant aboveground biomass included as a covariate. PERMANOVAs conducted on bacterial community structure (Weighted Unifrac) and root exudate profiles (Euclidean); significant *p*-values bolded ($p < 0.05$).

		F	*p*
Shannon Diversity	Treatment	5.71	0.003
	Focal Aboveground biomass	0.32	0.578
Pileau's Evenness	Treatment	0.59	0.675
	Focal Aboveground biomass	0.82	0.377
Chao1	Treatment	9.72	<0.001
	Focal Aboveground biomass	3.58	0.074
Bacterial community structure (all taxa)	Treatment	1.20	0.155
	Focal Aboveground biomass	0.72	0.802
Bacterial community structure (dominant taxa)	Treatment	1.08	0.32
	Focal Aboveground biomass	0.63	0.867
Bacterial community structure (non-dominant taxa)	Treatment	1.25	0.041; R^2 = 0.20
	Focal Aboveground biomass	1.08	0.315
Root Exudates	Treatment	4.32	<0.001, R^2 = 0.38
	Focal Aboveground biomass	3.84	0.006, R^2 = 0.08

Table 2.S2. PERMANOVA post-hoc pairwise *p-values* (FDR adjusted) for focal plant non-dominant bacterial community (lower 90% abundant) and all root exudates. ns denotes *p*-value > 0.10.

Non-dominant Bacterial Community				
	No neighbor	*A. gerardii*	*K. macrantha*	*R. hirta*
A. gerardii	ns	-	-	-
K. macrantha	ns	ns	-	-
R. hirta	0.075	0.098	0.09	-
P. virgatum	ns	ns	0.42	0.09
Root Exudates				
	No neighbor	*A. gerardii*	*K. macrantha*	*R. hirta*
A. gerardii	0.076	-	-	-
K. macrantha	ns	0.036	-	-
R. hirta	0.026	0.026	0.026	-
P. virgatum	0.030	0.153	0.026	0.026

Table 2.S3: Focal plant indicator bacterial genera by neighbor treatment. ‘A’ indicates the probability that the sample belongs to the identified treatment given the species in the sample (specificity); ‘B’ is the probability of finding the species in the sample belonging to that treatment (sensitivity). *p*-values are adjusted for false-discovery-rate. OTUs organized by stat value; a higher stat value means that the genera is more highly associated or enriched in that treatment.

Treatment	OTU	Taxonomy	A	B	stat	*p*
No neighbor	OTU3645	Bacteria; Planctomycetes; Phycisphaerae; Phycisphaerales; Phycisphaeraceae; AKYG587	0.667	1	0.816	0.027
	OTU1289	Bacteria; Chloroflexi; KD4-96; bacterium_Ellin6529; unclassified; unclassified	0.889	0.6	0.73	0.039
	OTU6331	Bacteria; Planctomycetes; Phycisphaerae; Phycisphaerales; unclassified; unclassified	0.571	0.8	0.676	0.027
	OTU1062	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Rhodomicrobium	0.384	1	0.619	0.035
	OTU188	Bacteria; Actinobacteria; Actinobacteria; Streptomycetales; Streptomycetaceae; Streptomyces	0.374	1	0.611	0.035
	OTU266	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingobium	0.298	1	0.545	0.035
A. gerardii	OTU7558	Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales; unclassified; unclassified	0.415	1	0.644	0.046
	OTU7030	Bacteria; Acidobacteria; Acidobacteria; Subgroup_3; unclassified; unclassified	0.364	1	0.603	0.045
	OTU2136	Bacteria; Proteobacteria; Deltaproteobacteria; Bdellovibrionales; Bdellovibrionaceae; OM27_clade	0.362	1	0.601	0.005
K. macrantha	OTU434	Bacteria; Proteobacteria; Gammaproteobacteria; Cellvibrionales; Cellvibrionaceae; Cellvibrio	0.496	1	0.704	0.005
	OTU1936	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Chitinimonas	0.42	1	0.648	0.035
	OTU432	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae; Rhizobium	0.395	1	0.628	0.027

Table 2.S3 (cont'd)

K. macrantha	OTU146	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Devosia	0.376	1	0.613	0.035
	OTU163	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; unclassified	0.351	1	0.593	0.008
	OTU180	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; unclassified	0.297	1	0.545	0.039
R. hirta	OTU183	Bacteria; Actinobacteria; Actinobacteria; Frankiales; unclassified; unclassified	0.605	1	0.778	0.008
	OTU17996	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomicrobium	1	0.6	0.775	0.027
	OTU1973	Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Zymomonas	0.947	0.6	0.754	0.017
	OTU425	Bacteria; Proteobacteria; Betaproteobacteria; Methylophilales; Methylophilaceae; Methylotenera	0.552	1	0.743	0.012
	OTU716	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Caulobacter	0.543	1	0.737	0.039
	OTU23254	Bacteria; Proteobacteria; Betaproteobacteria; Methylophilales; Methylophilaceae; Methylophilus	0.534	1	0.731	0.005
	OTU167	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Flavobacterium	0.525	1	0.724	0.039
	OTU207	Bacteria; Actinobacteria; Actinobacteria; Corynebacteriales; Nocardiaceae; Williamsia	0.632	0.8	0.711	0.027
	OTU162	Bacteria; Actinobacteria; Actinobacteria; Micrococcales; Micrococcaceae; Kocuria	0.493	1	0.702	0.048
	OTU3016	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; unclassified	0.479	1	0.692	0.013
	OTU29082	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Acidiphilium	0.462	1	0.679	0.045

Table 2.S3 (cont'd)

R. hirta	OTU1432	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae; Defluviicoccus	0.448	1	0.669	0.039
	OTU33279	Bacteria; Proteobacteria; Betaproteobacteria; Methylophilales; Methylophilaceae; unclassified	0.425	1	0.652	0.039
	OTU1029	Bacteria; Planctomycetes; Phycisphaerae; WD2101_soil_group; unclassified; unclassified	0.303	1	0.55	0.008
	OTU629	Bacteria; Planctomycetes; Planctomycetacia; Planctomycetales; Planctomycetaceae; Pirellula	0.292	1	0.54	0.039
	OTU1819	Bacteria; Planctomycetes; Planctomycetacia; Planctomycetales; Planctomycetaceae; Gemmata	0.286	1	0.535	0.025
	OTU47	Bacteria; Planctomycetes; Planctomycetacia; Planctomycetales; Planctomycetaceae; Planctomyces	0.259	1	0.508	0.017
P. virgatum	OTU4888	Bacteria; Proteobacteria; Deltaproteobacteria; Desulfuromonadales; GR-WP33-58; unclassified	0.625	1	0.791	0.045
	OTU1821	Bacteria; Cyanobacteria; Cyanobacteria; unclassified; unclassified; unclassified	0.616	1	0.785	0.017
	OTU4965	Bacteria; Armatimonadetes; Armatimonadia; Armatimonadales; unclassified; unclassified	0.539	1	0.734	0.008
	OTU15612	Bacteria; Actinobacteria; Actinobacteria; Kineosporiales; Kineosporiaceae; unclassified	0.448	1	0.67	0.027
	OTU1161	Bacteria; Actinobacteria; Actinobacteria; Micromonosporales; Micromonosporaceae; unclassified	0.3361	1	0.58	0.04
	OTU1251	Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales; Coxiellaceae; Aquicella	0.286	1	0.534	0.046

Table 2.S4. Distance-based Redundancy Analysis (Weighted Unifrac) results for the dominant taxa (top 10% most abundant OTUs) and non-dominant-taxa (lower 90% of OTUs). Significant *p*-values are bolded ($p < 0.05$).

	Dominant bacterial taxa		Non-dominant bacterial taxa	
	p	R^2	*p*	R^2
Malic Acid	0.026	0.087	0.016	0.072
Mean Shoot CN	0.029	0.075	0.682	0.036
Stearic Acid	0.116	0.058	0.098	0.057
Overall model significance	$F_{3,20} = 1.81$, $p = 0.005$		$F_{3,20} = 1.32$, $p = 0.041$	

Table 2.S5. Significant correlations between exudates and bacterial genera using the nc.score of co-occurrence. Q-value is equivalent to FDR corrected *p*-values.

OTU	Metabolite	nc	q	Phylum	Class	Order	Family	Genus
OTU 1850	4-Pyridoxate	0.54	0.047	Planctomycetes	Phycisphaerae	CPla-3_termite_group	unclassified	unclassified
OTU 23254	Fumaric Acid	0.54	0.012	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	Methylophilus
OTU 23254	Malic Acid	0.50	0.016	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	Methylophilus
OTU 23254	Pyruvic Aldehyde	0.58	0.001	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	Methylophilus
OTU 2415	Citramalic Acid	0.57	0.022	Proteobacteria	Alphaproteobacteria	Rickettsiales	SM2D12	unclassified
OTU60	Inosine	0.54	0.013	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	unclassified

Table 2.S6. Indicator bacterial genera by soil incubation treatment. A indicates the probability that the sample belongs to the identified treatment given the species in the sample (specificity); B is the probability of finding the species in the sample belonging to that treatment (sensitivity). *p*-values are adjusted for false-discovery-rate. OTUs organized by stat value; a higher stat value means that the genera is more highly associated or enriched in that treatment.

Treatment	OTU	Taxonomy	A	B	stat	*p*
Low Malic Acid	OTU3377	Bacteria; Proteobacteria; Deltaproteobacteria; Bdellovibrionales; Bacteriovoracaceae; Peredibacter	0.92	1	0.957	0.028
	OTU1369	Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales; Legionellaceae; unclassified	0.86	1	0.926	0.028
	OTU756	Bacteria; Planctomycetes; Phycisphaerae; WD2101_soil_group; Planctomycetales_bacterium_Ellin7244; unclassified	0.83	1	0.913	0.039
	OTU1781	Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; Sandaracinaceae; Sandaracinus	0.83	1	0.913	0.028
	OTU3188	Bacteria; Proteobacteria; Gammaproteobacteria; Chromatiales; Ectothiorhodospiraceae; Acidiferrobacter	0.82	1	0.905	0.049
	OTU2054	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Beijerinckiaceae; unclassified	0.81	1	0.901	0.05
	OTU2100	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Rhodobacter	0.79	1	0.89	0.028
	OTU2931	Bacteria; Acidobacteria; Acidobacteria; Subgroup_4; unclassified; unclassified	0.79	1	0.889	0.042
	OTU1062	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Rhodomicrobium	0.79	1	0.889	0.028
	OTU1190	Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; Polyangiaceae; Polyangium	0.77	1	0.875	0.049
	OTU609	Bacteria; Chloroflexi; Ktedonobacteria; JG30-KF-AS9; unclassified; unclassified	0.94	0.8	0.869	0.045
	OTU1694	Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; Polyangiaceae; unclassified	0.75	1	0.866	0.05

Table 2.S6 (cont'd)

Treatment	OTU	Taxonomy	A	B	stat	*p*
	OTU29082	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Acetobacteraceae; Acidiphilium	0.75	1	0.866	0.049
	OTU30414	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; MNG7; unclassified	0.74	1	0.859	0.028
	OTU103	Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; unclassified; unclassified	0.74	1	0.858	0.028
	OTU1357	Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales; SM2D12; unclassified	0.73	1	0.857	0.042
	OTU5959	Bacteria; Proteobacteria; Gammaproteobacteria; NKB5; unclassified; unclassified	0.73	1	0.855	0.045
	OTU5275	Bacteria; Bacteroidetes; Sphingobacteriia; unclassified; unclassified; unclassified	0.9	0.8	0.851	0.042
Low Malic Acid	OTU1364	Bacteria; Acidobacteria; Holophagae; unclassified; unclassified; unclassified	0.72	1	0.849	0.042
	OTU3731	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Devosia	0.71	1	0.84	0.028
	OTU412	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; JG34-KF-361; unclassified	0.68	1	0.828	0.042
	OTU1368	Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; Polyangiaceae; Sorangium	0.69	1	0.828	0.028
	OTU6765	Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; KD3-10; unclassified	0.68	1	0.822	0.045
	OTU96	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; KF-JG30-B3; unclassified	0.68	1	0.822	0.028
	OTU80	Bacteria; Acidobacteria; Holophagae; Subgroup_10; ABS-19; unclassified	0.66	1	0.815	0.028
	OTU310	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae; Dongia	0.66	1	0.813	0.028

Table 2.S6 (cont'd)

Treatment	OTU	Taxonomy	A	B	stat	*p*
	OTU92	Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; Haliangiaceae; Haliangium	0.65	1	0.808	0.042
	OTU3131	Bacteria; Proteobacteria; Deltaproteobacteria; Bdellovibrionales; Bdellovibrionaceae; Bdellovibrio	0.64	1	0.798	0.028
	OTU730	Bacteria; Planctomycetes; OM190; unclassified; unclassified; unclassified	0.62	1	0.79	0.049
	OTU723	Bacteria; Planctomycetes; Pla4_lineage; unclassified; unclassified; unclassified	0.62	1	0.788	0.042
	OTU195	Bacteria; Acidobacteria; Holophagae; Subgroup_7; unclassified; unclassified	0.62	1	0.784	0.028
	OTU193	Bacteria; Acidobacteria; Acidobacteria; Subgroup_3; Unknown_Family; Candidatus_Solibacter	0.61	1	0.781	0.049
Low Malic Acid	OTU1311	Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae_[Subgroup_1]; Candidatus_Koribacter	0.61	1	0.781	0.042
	OTU113	Bacteria; Acidobacteria; Subgroup_22; unclassified; unclassified; unclassified	0.6	1	0.776	0.045
	OTU325	Bacteria; Latescibacteria; unclassified; unclassified; unclassified; unclassified	0.6	1	0.774	0.042
	OTU8999	Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae_[Subgroup_1]; unclassified	0.59	1	0.769	0.049
	OTU19	Bacteria; Acidobacteria; Acidobacteria; Subgroup_4; RB41; unclassified	0.58	1	0.76	0.039

Table 2.S6 (cont'd)

Treatment	OTU	Taxonomy	A	B	stat	*p*
High Malic Acid	OTU327	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Brevundimonas	1	1	1	0.039
	OTU959	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Sphingobacteriaceae; Pedobacter	0.99	1	0.993	0.05
	OTU1937	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Pseudoxanthomonas	0.94	1	0.97	0.039
	OTU11690	Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales; Oceanospirillaceae; Pseudospirillum	0.92	1	0.957	0.042
	OTU911	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Hyphomonadaceae; Hirschia	0.88	1	0.938	0.045
	OTU1414	Bacteria; Acidobacteria; Acidobacteria; Subgroup_3; Unknown_Family; unclassified	0.81	1	0.901	0.05
	OTU2822	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae; Flavitalea	0.8	1	0.894	0.045
	OTU520	Bacteria; Bacteroidetes; Cytophagia; Cytophagales; Cytophagaceae; Chryseolinea	0.79	1	0.89	0.039
	OTU33743	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae; Parasegetibacter	0.79	1	0.886	0.028
	OTU467	Bacteria; Acidobacteria; Acidobacteria; Subgroup_3; SJA-149; unclassified	0.72	1	0.847	0.05
	OTU40	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; unclassified	0.71	1	0.843	0.05

Table 2.S6 (cont'd)

Treatment	OTU	Taxonomy	A	B	stat	*p*
High Malic Acid	OTU2074	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Sphingobacteriaceae; unclassified	0.7	1	0.836	0.045
Low Malic Acid	OTU1142	Bacteria; Fibrobacteres; Fibrobacteria; Fibrobacterales; Fibrobacteraceae; unclassified	0.64	1	0.8	0.045
	OTU34	Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadales_Incertae_Sedis; Steroidobacter	0.64	1	0.798	0.039
	OTU2060	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; NS11-12_marine_group; unclassified	0.63	1	0.795	0.028
	OTU93	Bacteria; Proteobacteria; Betaproteobacteria; SC-I-84; unclassified; unclassified	0.63	1	0.791	0.045
	OTU2556	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Rhodospirillales_Incertae_Sedis; Reyranella	0.62	1	0.788	0.049
	OTU280	Bacteria; Actinobacteria; Acidimicrobiia; Acidimicrobiales; unclassified; unclassified	0.61	1	0.782	0.049
	OTU31	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Xanthobacteraceae; Variibacter	0.59	1	0.77	0.039
	OTU171	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; unclassified; unclassified	0.58	1	0.762	0.039
	OTU13827	Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae; Parafilimonas	0.58	1	0.76	0.028
	OTU47	Bacteria; Planctomycetes; Planctomycetacia; Planctomycetales; Planctomycetaceae; Planctomyces	0.58	1	0.759	0.028

Table 2.S6 (cont'd)

Treatment	OTU	Taxonomy	A	B	stat	*p*
Low Malic Acid	OTU837	Bacteria; Proteobacteria; Betaproteobacteria; unclassified; unclassified; unclassified	0.57	1	0.752	0.028

Table 2.S7. Statistical packages used in R for analyses. * Indicates that more information on statistical parameters or analysis is provided below the table.

Dataset	Analysis	Package	Function	Citation
Univariate Analyses				
Plant, soil, bacterial alpha diversity	1-factor ANOVA	Car	lm	(Fox and Weisberg, 2011)
Plant, soil, bacterial alpha diversity	1-factor ANOVA post-hoc comparisons	Emmeans	Emmeans	(Lenth, 2019)
All	All visualization graphics	Ggplot2	Ggplot2	(Wickham, 2016)
Multivariate Analyses				
Bacterial community	Microbial data filtering	Phyloseq	Several, including tax_glom, merge_samples, etc.	(McMurdie and Holmes, 2013)
Bacterial community	Taxa differential abundance	ANCOMBC	Ancombc	(Lin and Peddada, 2020)
Bacterial community	Indicator Species Analysis*	Indicspecise	Multiplatt	(Caceres and Legendre, 2009)
Bacterial community	Distance-based Redundancy Analysis*	Vegan	dbrda, ordistep (for model selection)	(Oksanen et al., 2018)
Bacterial community & root exudates	PERMANOVA*	Vegan	Adonis2	(Oksanen et al., 2018)
Bacterial community & root exudates	PERMANOVA post-hoc comparisons	RVAideMemoire	Pairwise.perm.manova	(Hervé, 2015)
Bacterial community & root exudates	Matrix Similarity Analysis	Vegan	Protest	(Oksanen et al., 2018)
Bacterial community & root exudates	Variance Partitioning Analysis	Vegan	Varpart	(Oksanen et al., 2018)
Bacterial community & root exudates	Correlation among bacterial genera and identified metabolites*	CCREPE	ccrepe	(Schwager et al., 2014)
Root exudates	sPLS-DA*	MixOmics	tune.splsda, perf	(Rohart et al., 2017)
Root exudates	Pearson Correlations	Hmisc	Rcorr	(Harrell and Dupont, 2021)
Root exudates	Correlation heatmap visualization	Superheat	superheat	(Barter and Yu, 2018)

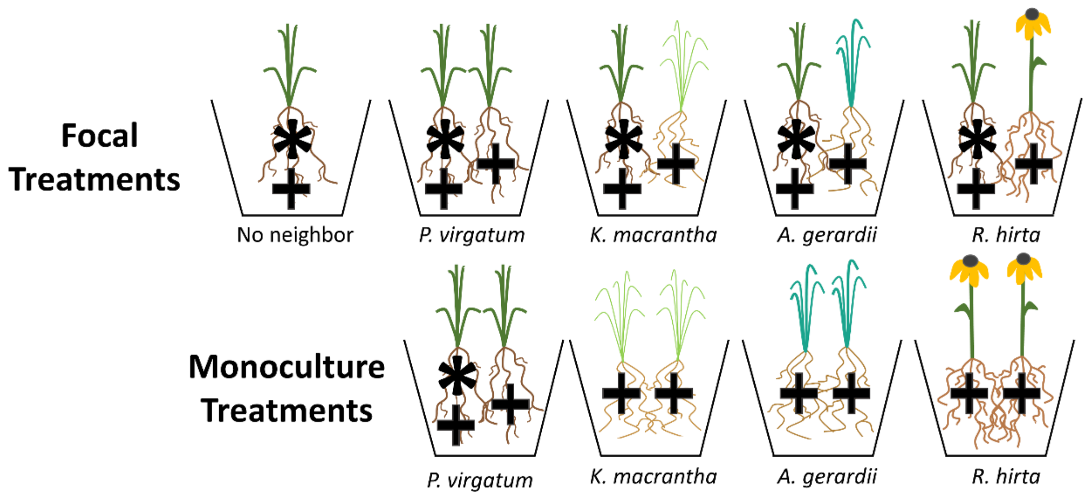


Figure 2.S1. Experimental design showing five focal plant treatments and four monoculture treatments. *P. virgatum* is always the focal plant, and the *P. virgatum* neighbor treatment was also used for statistical comparisons among monocultures. Asterix symbol (*) denotes plants used for root exudate collection; Plus sign (+) indicates roots sampled for rhizosphere bacterial community analysis.

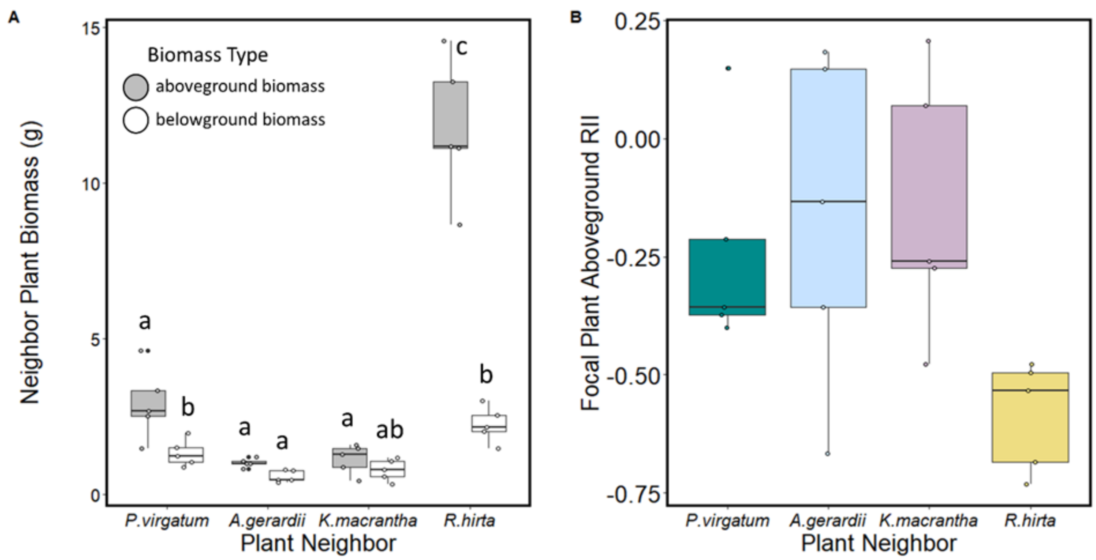


Figure 2.S2. A) neighbor plant aboveground (gray boxes) and belowground (white boxes) biomass in focal plant treatments; B) Relative strength of competition index for focal plant aboveground biomass. The central line is the median value, vertical bars represent the first and third quartile, and dots represent individual replicate values. Different letters denote significant differences among treatments (false discovery rate, p < 0.05); for Figure A, the letters are grouped within biomass type.

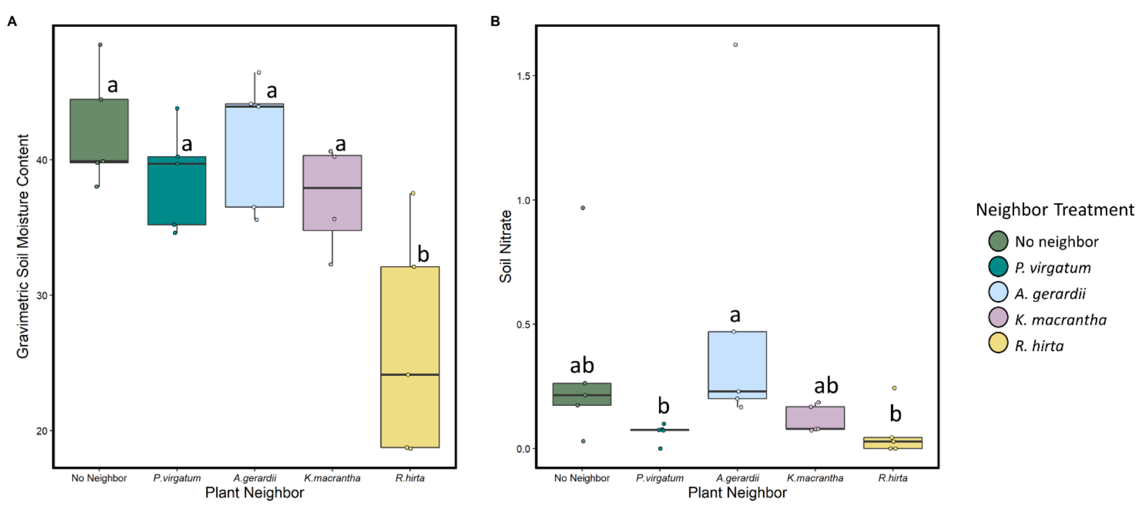


Figure 2.S3. Focal plant treatment A) gravimetric soil moisture content (g water g^{-1} dry soil) and b) soil nitrate (µg nitrate g^{-1} dry soil) at the pot-level. The central line is the median value, vertical bars represent the first and third quartile, and dots represent individual replicate values. Different letters denote significant differences among treatments (false discovery rate, *p* < 0.05).

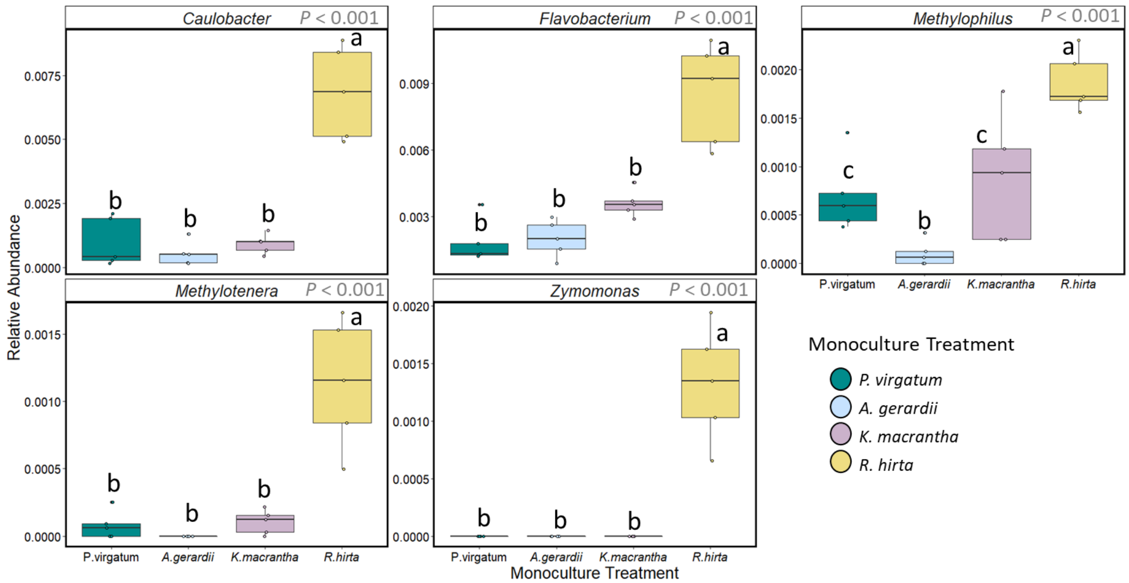


Figure 2.S4. Relative abundance of bacterial genera identified as indicators when the focal neighbored *R. hirta* grouped by their presence in monoculture treatments. The central line is the median value, vertical bars represent the first and third quartile, and dots represent outliers. *p*-value from 1-way ANOVA presented; different letters denote significant differences among treatments (false discovery rate, $p < 0.05$).

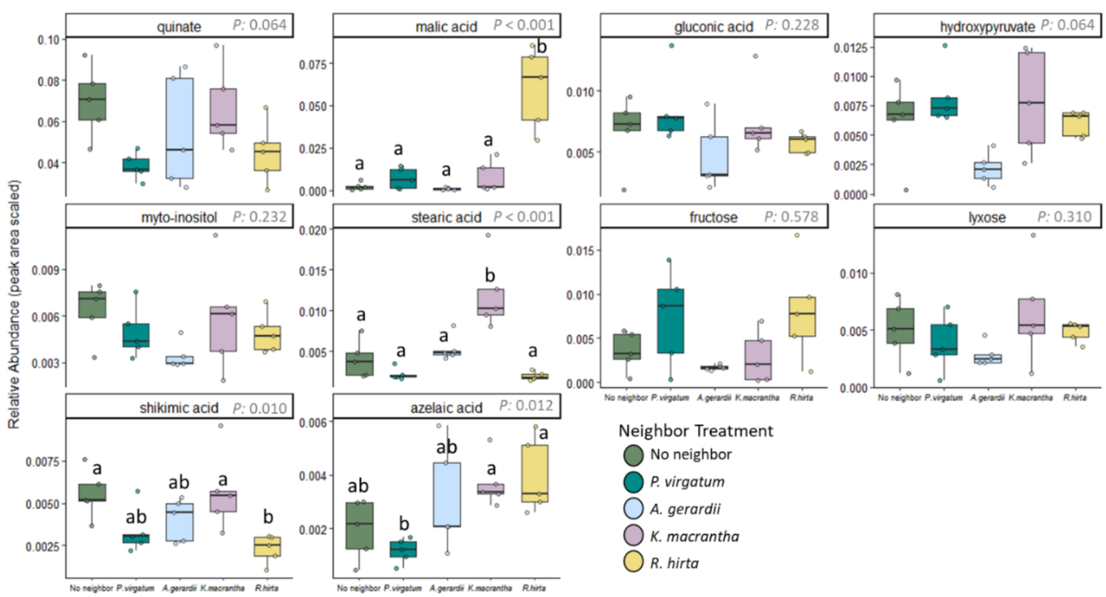


Figure 2.S5. Relative abundance (peak area scaled) of top 10 most abundant identified exudates by focal plant treatment. The central line is the median value, vertical bars represent the first and third quartile, and dots represent individual replicate values. *p*-value from 1-way ANOVA presented; different letters denote significant differences among treatments (false discovery rate, $p < 0.05$).

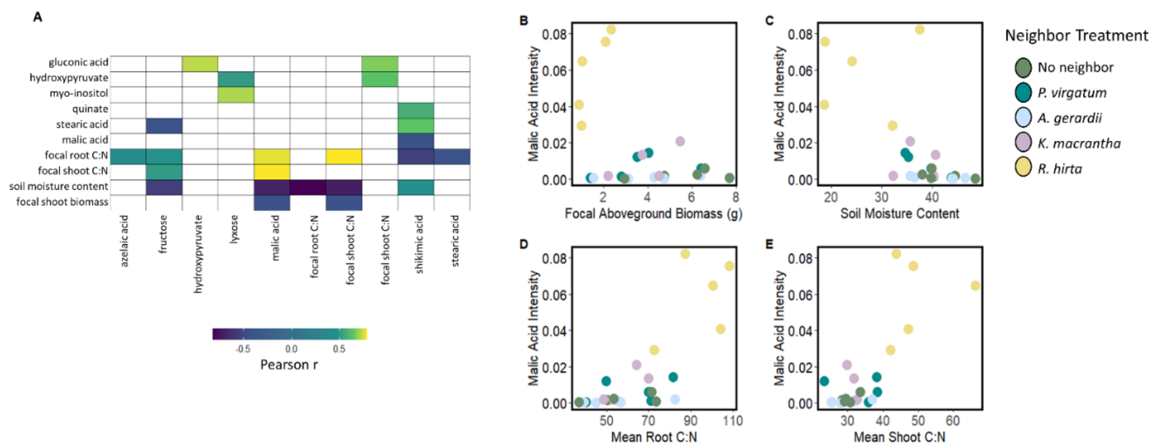


Figure 2.S6. A) Significant Pearson correlation ($p < 0.05$) between plant and soil factors and top 10 most abundant identified exudates. Color scale indicates strength of correlation (r, purple = negative; yellow = positive). Blank spaces indicate no significant correlation. B-E) Scatterplot showing relationship between malic acid and plant and soil factors, colored by neighbor treatment.

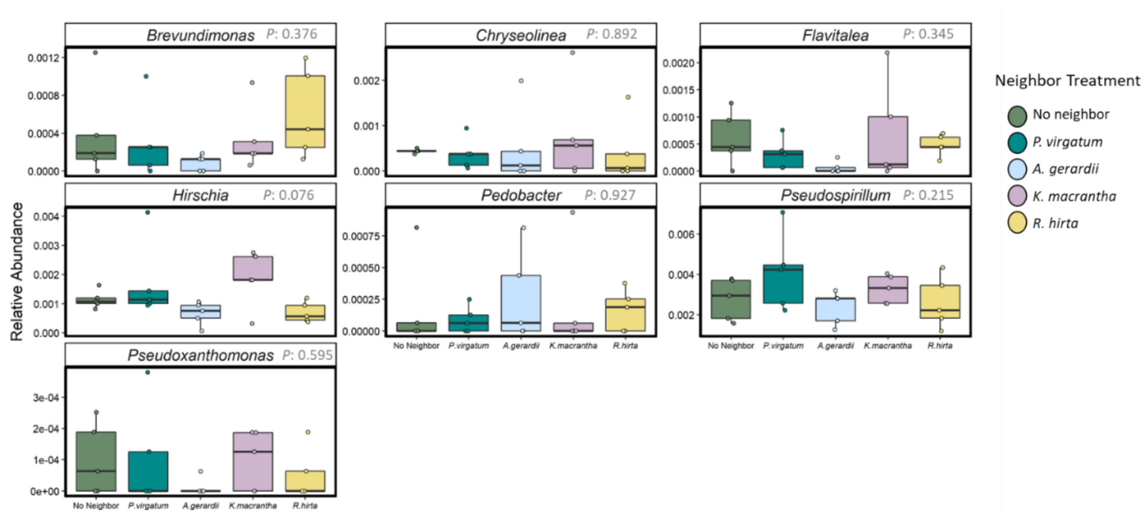


Figure 2.S7. Relative abundance of bacterial genera enriched in high malic acid soil incubation (ANCOMBC differential abundance analysis), plotted by their relative abundance in greenhouse focal treatments. The central line is the median value, vertical bars represent the first and third quartile, and dots represent individual replicate values. p-value from 1-way ANOVA presented.

APPENDIX C: CHAPTER THREE SUPPLEMENTAL METHODS AND RESULTS

RHIZOBOX DESIGN, SOIL, AND PLANTING

The boxes (54 cm wide x 30 cm tall x 4 cm deep) were made of white High-Density Polyethylene (HDPE, back of box) and clear polycarbonate resin (front of box), fastened together with metal nuts and bolts and all-purpose cement (Figure 3.S1). The boxes were split into three sections with hollow, acrylic square columns that provided additional structural support. Foam was glued around all edges of the rhizobox to form a tight, waterproof seal between the box edges and the front cover. The boxes were placed at a 60° angle from the ground in the greenhouse to encourage root growth along the clear side of the box for easier sampling. White HDPE was chosen for the back of boxes to minimize increasing the temperature of the soil; clear polycarbonate was chosen as the front panel to allow root tracking throughout the course of the experiment without opening the rhizobox, but this was covered with black plastic throughout the experiment to avoid light exposure to the roots.

Each rhizobox was filled to a consistent bulk soil density and water content (1.28 g/cm3 and 20% volumetric water content). To do so, the fronts of the rhizoboxes were removed and dry, sieved soil and water were incrementally added to each compartment to ensure even moisture distribution. The two hollow columns in each rhizobox were also filled to the equivalent soil moisture and bulk density. After filling the rhizoboxes, one week old seedlings were transplanted into each of the three sections. The seedlings were established in potting media (Suremix) with 5% dry field soil by volume (seed source: Native Connections, Kalamazoo, Michigan, USA). They were continually replaced for up to two weeks if they died, and then grew for 14 weeks in their neighbor treatments with temperatures controlled at a maximum of 29 °C during the day and minimum of 20 °C at night with 16 hours of artificial lighting. They were watered with RO water as needed and fertilized twice during the experiment to adjust for potassium deficiency in the soil (Monopotassium phosphate to the equivalent of 56.04 Kg P Ha^{-1}).

ROOT EXUDATES LC-MS

The reconstituted (80% methanol) root exudate samples were injected into a Waters Acquity UPLC HSS-T3 column (2.1x100 mm) and compounds were separated using the following gradient: initial conditions were 100% mobile phase A (10 mM ammonium formate in water, pH 3) and 0% mobile phase B (acetonitrile), hold at 0% B until 1.0 min, linear ramp to 99% B at 7.0 min, hold at 99% B until 8.0 min, return to 0% B at 8.01 min and hold until 10 min. The flow rate was 0.3 ml/min and the column temperature was held at 40°C. Compounds were ionized with electrospray ionization operating in either positive (capillary voltage 3.0 kV) or negative ion mode (capillary voltage 2.0 kV). Cone voltage was set to 30 V, source temperature was 100°C, desolvation temperature was 350°C, cone gas flow was 40 L/hr, and desolvation gas flow was 600 L/hr. Mass spectra were acquired using a data-independent MSE method over a mass range of m/z 50-1500 with separate acquisition functions for scans (0.2 seconds/scan) with no collision energy and scans (0.2 seconds/scan) with a collision energy ramp of 20-80V. Lockmass correction was performed using leucine enkephalin as the reference compound. Peak alignment and picking were performed using Progenesis QI software (Nonlinear Dynamics, Waters) with a pooled sample used as the alignment reference.

DNA LIBRARY PREPARATION

For library preparation, the fungal (ITS) DNA was amplified with DreamTaq Green DNA polymerase (Thermo Scientific) and the bacterial DNA (16S) was amplified with Platinum Taq DNA polymerase (Thermo Scientific). We used a modified version of a three-step PCR protocol, which was previously described (Lundberg et al., 2013a) and used (Benucci et al., 2020; da Costa et al., 2022). First, the genomic DNA was amplified with generic primers (step 1, 10 PCR cycles) to enrich for the fungal rDNA template, and then the PCR products were amplified with primers incorporating 1- to 6-nucleotide frameshifts to increase diversity between samples (step 2, 10 PCR cycles). Finally, we incorporated 10-nucleotide indexing barcodes and Illumina adapters to the PCR products (step 3, 15 PCR cycles). We assessed the PCR size and concentration of the PCR products with a QIAxcel Advanced machine with a DNA Fast Analysis kit (Qiagen). Sample libraries were then normalized with a SequalPrep normalization plate kit (Thermo Fisher Scientific) and pooled. The generated amplicon library was then concentrated at approximately 20:1 with Amicon Ultra 0.5-mL 50K filters (EMDmillipore, Germany) and purified from primer dimers with Agencourt AMPure XP magnetic beads (Beckman Coulter, USA). The final multiplexed libraries were sequenced on an Illumina MiSeq analyzer using the v3 600 cycle kit (Illumina, USA) by the MSU Research Technology Support Facility.

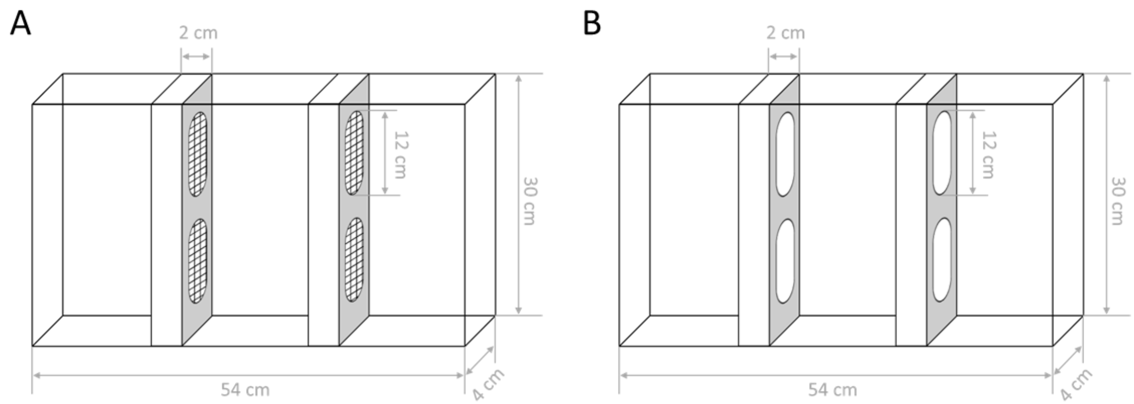


Figure 3.S1. Design and dimension of rhizoboxes. A) shows rhizobox with Barrier that prevented active root interactions, while allowing mycorrhizal interactions (35 μM mesh barrier); B) shows rhizobox with No Barrier that allowed active root interactions. Columns in the center were hollow, filled with soil, and used to separate each of the three sections and provide structural stability. Roots could grow through the column in the No Barrier boxes (B), but not in the boxes with mesh covering the opening (A).

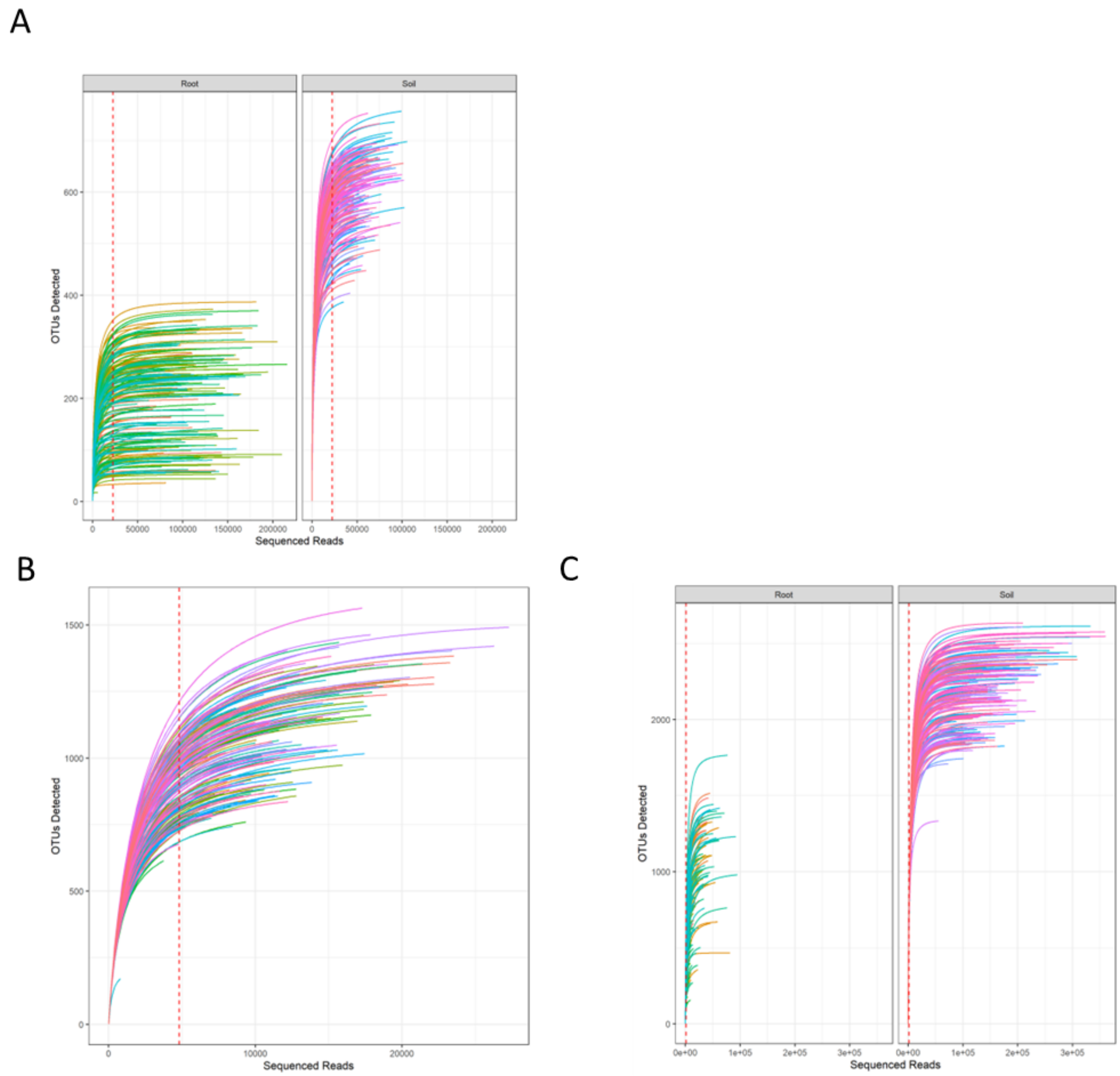


Figure 3.S2. Microbiome rarefaction curves for three datasets used for analyses: combined fungal root-associated and rhizosphere soil (A), bacterial rhizosphere soil only(B), and combined bacterial root-associated and rhizosphere soil communities(C). Each line represents a unique sample; the red-dashed line represents the minimum sample read depth used for rarefying in each dataset. All fungal analyses were conducted on the combined dataset (A), which was rarefied to 22,547 reads. The rhizosphere soil bacterial dataset (B) was rarefied to 4,814 reads and used for all rhizosphere soil bacterial analyses. The combined bacterial dataset (C), which was rarefied to 1,444 reads, was used for the root-associated bacterial analyses and analyses that looked at differences between the two rhizosphere compartments. Rarefaction plots were made after filtering contaminants, low-read OTUs and low-coverage samples.

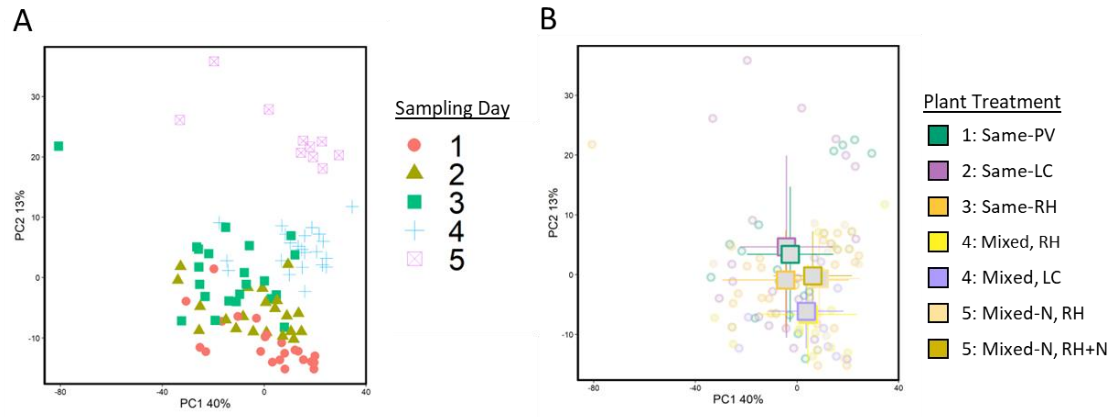


Figure 3.S3. Focal plant root exudates plotted by sampling day (A) and by neighbor treatment (B), Euclidean Distance, PCA.

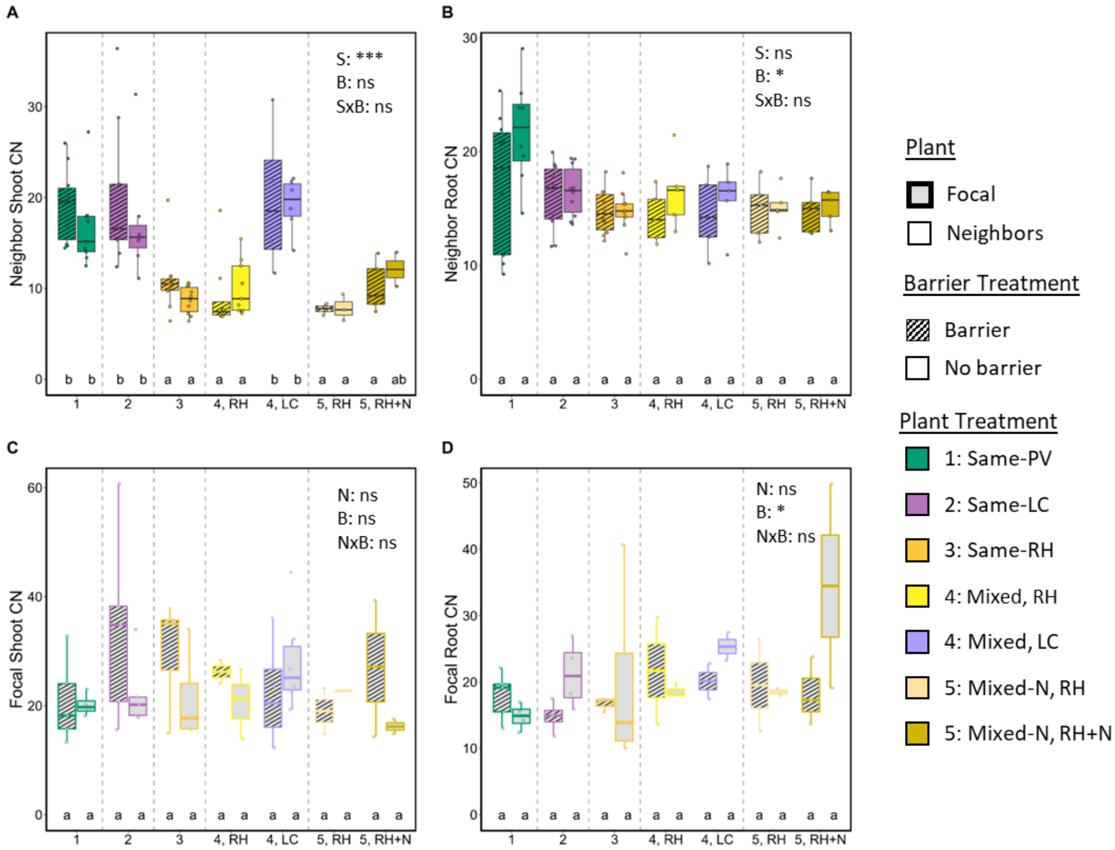


Figure 3.S4. Variation in neighbor plant shoot and root CN (A,B) and focal plant shoot and root CN (C,D). Color and number denote neighbor treatment. Vertical gray lines separate samples present within a single treatment. Striped boxes represent barrier treatment (no root interactions) and solid boxes represent the no barrier treatment (active root interactions). The central line is the median value for each plant, vertical bars represent the first and third quartiles of the data, raw data shown in points. Different letters denote significant differences among all plants and barriers (false discovery rate, p < 0.05). ANOVA results in upper right corner of each panel denote significant p-value for Neighbor (N), Barrier (B), Neighbor by Barrier interaction (NxB) for focal plants(Panels A-C) and Species (S), Barrier (B), and Species by Barrier (SxB) interaction for neighbor plants (Panels D-F) ; significance values: ns $p > 0.10$, + $p < 0.10$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

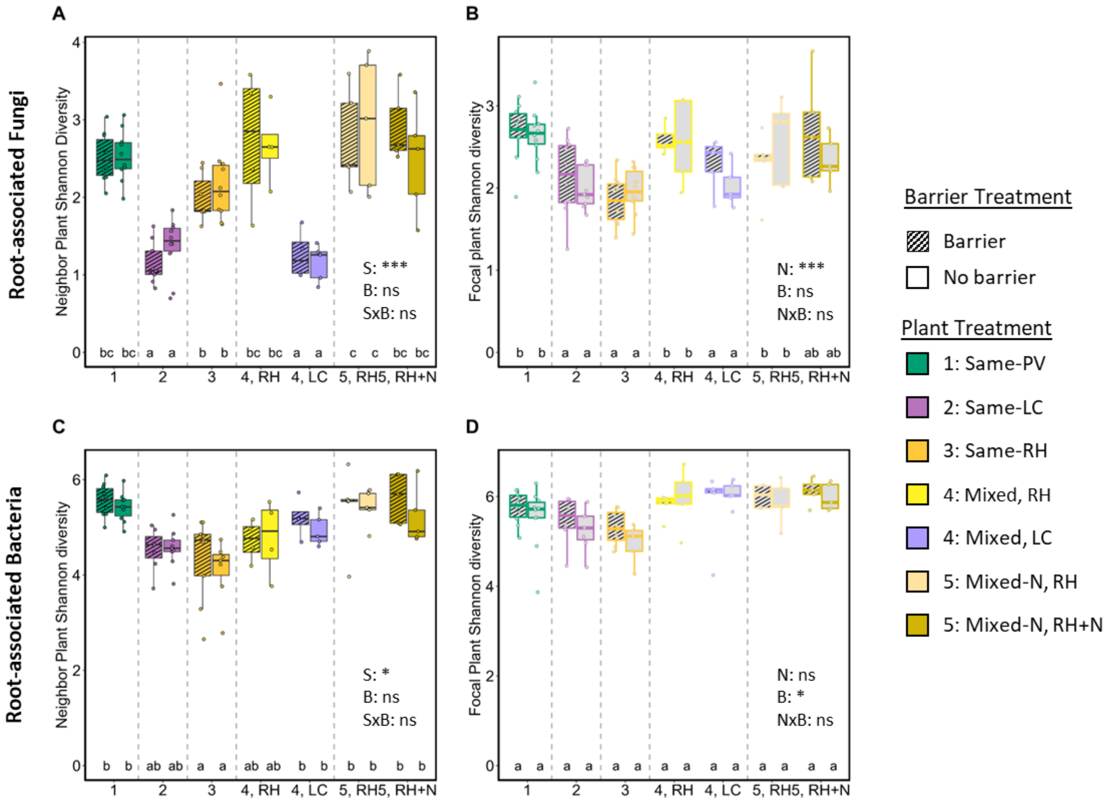


Figure 3.S5. Root-associated microbiome Shannon diversity for neighbor plant fungi (A), focal plant fungi (B), neighbor plant bacteria (C), and focal plant bacteria (D). Color and number denote neighbor treatment; neighbor plant boxplots are filled with the treatment color; focal plant boxplots are gray and outlined in neighbor treatment color. Vertical gray lines separate samples present within a single treatment. Striped boxes represent barrier treatment (no root interactions) and solid boxes represent the no barrier treatment (active root interactions). The central line is the median value for each plant, vertical bars represent the first and third quartiles of the data, raw data shown in points. Different letters denote significant differences among all plants and barriers (false discovery rate, $p < 0.05$). ANOVA results in lower right corner of each panel denote significant p-value for Neighbor (N), Barrier (B), Neighbor by Barrier interaction (NxB) for focal plants (Panels A-C) and Species (S), Barrier (B), and Species by Barrier (SxB) interaction for neighbor plants (Panels D-F) ; significance values: ns $p > 0.10$, + $p < 0.10$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

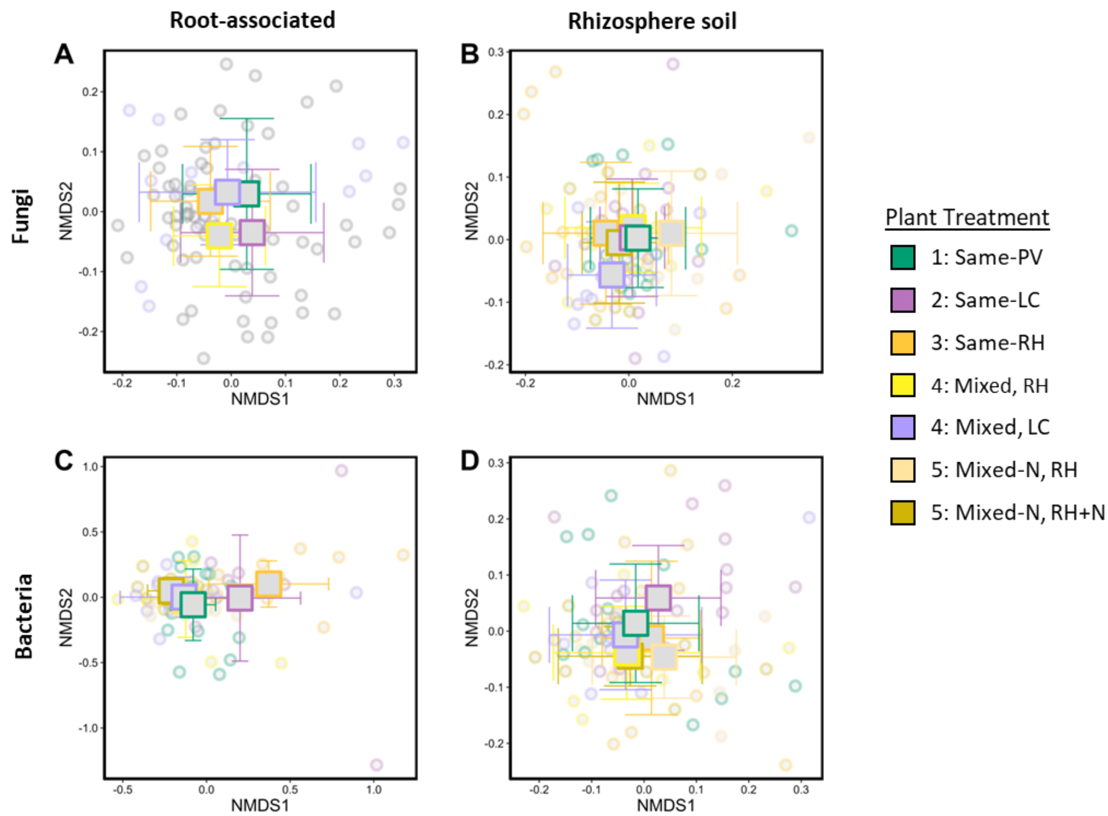


Figure 3.S6. NMDS ordinations (Bray-Curtis) of focal plant microbiomes for root-associated fungi (A), rhizosphere soil fungi (B), root-associated bacteria (C), and rhizosphere soil bacteria(D). Focal plants are filled with gray and border color represents their neighbor. Barrier and No Barrier samples are combined within each treatment. Large squares represent centroid of all sample points and bars represent ± 1 SD from the centroid mean. See Table 3.2 for statistical results.

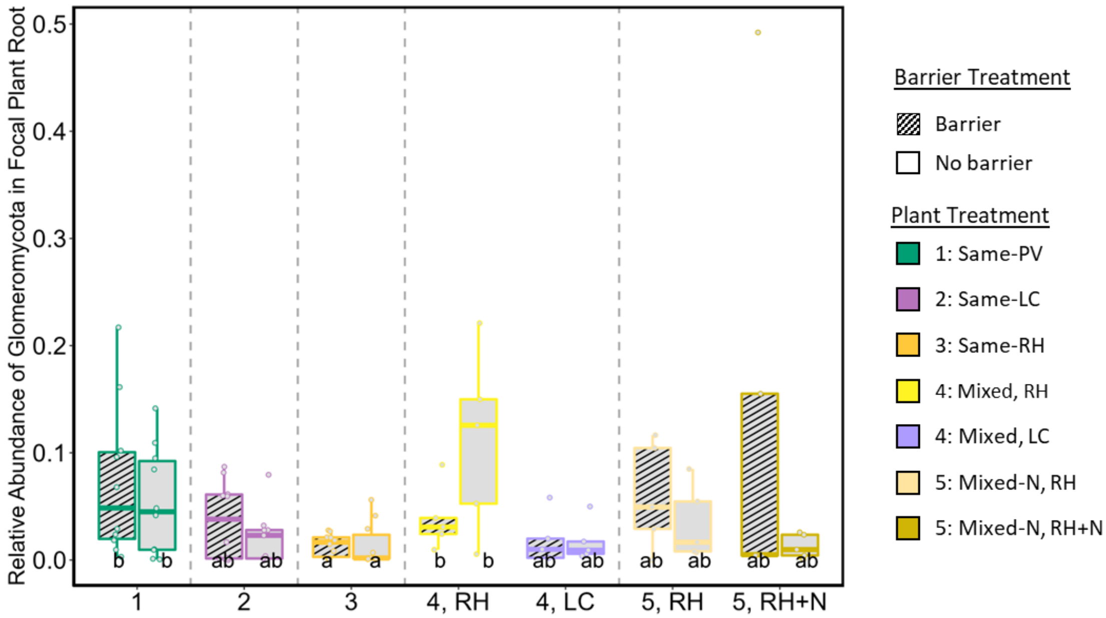


Figure 3.S7. Percentage of the focal plant's microbiome reads that are shared with its direct neighbor for fungal root-associated community (A), fungal rhizosphere soil community (B), bacterial root-associated community (C) and bacterial rhizosphere soil community (D). Colored border represent neighbor treatment. Vertical gray lines separate samples present within a single treatment. Striped boxes represent barrier treatment (no root interactions) and solid boxes represent the no barrier treatment (active root interactions). The central line is the median value for each plant, vertical bars represent the first and third quartiles of the data, raw data shown in points. Different letters denote significant differences among all plants and barriers (false discovery rate, $p < 0.05$). ANOVA within the lower right of each panel denote significant *p*-value for Neighbor Treatment (N), Barrier (B), Neighbor by Barrier interaction (NxB); significance values: ns $p > 0.10$, + $p < 0.10$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

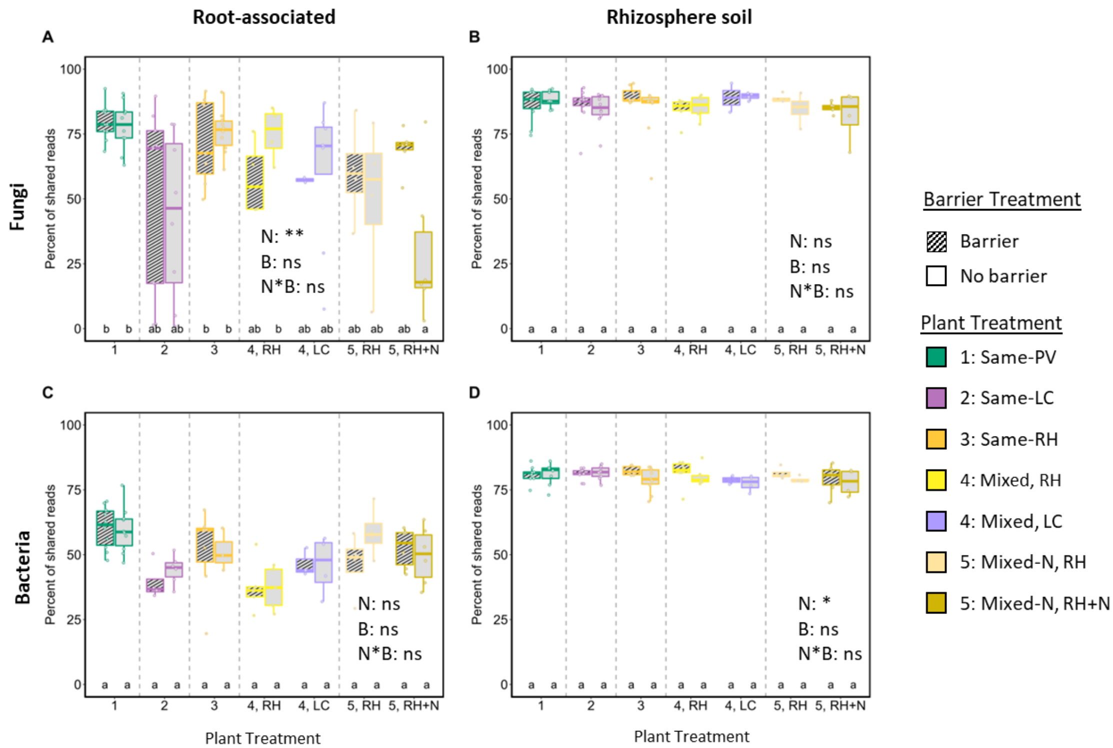


Figure 3.S8. Percentage of the focal plant's microbiome reads that are shared with its direct neighbor for the root-associated fungi (A), rhizosphere soil fungi (B), root-associated bacteria (C) and rhizosphere soil bacteria (D). Colored border represent neighbor treatment. Vertical gray lines separate samples present within a single treatment. Striped boxes represent barrier treatment (no root interactions) and solid boxes represent the no barrier treatment (active root interactions). The central line is the median value for each plant, vertical bars represent the first and third quartiles of the data, raw data shown in points. Different letters denote significant differences among all plants and barriers (false discovery rate, $p < 0.05$). ANOVA results within the lower right of each panel denote significant p-value for Neighbor Treatment (N), Barrier (B), Neighbor by Barrier interaction (NxB); significance values: ns $p > 0.10$, + $p < 0.10$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

APPENDIX D: CHAPTER FOUR SUPPLEMENTAL METHODS AND RESULTS

METHODOLOGICAL CONSIDERATIONS

Mental models have been studied through many different approaches and each have their advantages and disadvantages (Jones et al., 2011). The mental modelling approach we used is most similar to fuzzy cognitive mapping (FCM) methods, whereby participants identify variables that influence a system or concept (e.g. soil health) and then depict connections between those factors using visual methods (Gray et al., 2014). The benefit of this approach is that participants can illustrate and interact with their own representation of their mental model (Bardenhagen et al., 2020), which is in contrast to indirect elicitation approaches that extract mental models from texts and interviews (Halbrendt et al., 2014; Hoffman et al., 2014). FCM methods often gather quantitative data by asking participants to numerically rank relationships between factors (e.g. +1 to -1) (Özesmi and Özesmi, 2004), but we did not do this because our main objective was to identify the terms central to farmers' understanding of soil health, rather than the degree to which the terms influence one another. We also chose to allow participants to populate their mental models with freely chosen concepts, rather than using the same standardized concepts for all participants. While a more free-form approach can be biased by the researchers' subjective categorization and aggregation during analysis, it is less likely to limit participants perceptions and to stifle potentially interesting heterogeneity in their responses (Gray et al., 2014).

Table 4.S1. Terms included in farmers' soil health mental models, which were aggregated at two-levels, by topic and category. Topics are sorted in order of frequency (see Figure 4.4). No topics categorized as "crop" were included in the soil health mental models.

Topic	Category	Terms Aggregated
Organic Matter	Soil	"organic matter", "humus", "organic material", "carbon content", "soil doesn't crust over on top with sunshine (higher OM don't crust as easy, more forgiving to machine abuse)", "low CEC and organic matter levels (least productive too much large sand particles to hold onto nutrients, less particles to hold onto nutrients vs. clay)", "high CEC levels and organic levels",
Compaction	Soil	"compaction", "compaction (big contributor to bad health)", "compaction (tillage?)"
Soil Biology	Soil	"high microbial activity and root decaying matts (more air and food to feed roots) ", "earth worms", "microorganisms", "microbe availability ", "biologicals", "good bugs", "microorganisms (pollution?)", "soil microbes at work ", "microbe activity", "biological activity"
Tillage	Practice	"tillage", "overuse of tillage (also breaking down soil particles)", "no-till", "Tillage timing and method", "type of tillage", "tillage (double edged sword)"
Fertility/Nutrients	Soil	"available nutrients", "fertility", "precision nutrient management", "nutrients", "sufficient nutrients", "micro-nutrients", "fertility (good balance needed for good soil health)", "good nutrient levels (fertilize?)", "good fertility"
Drainage	Soil	"drainage", "good drainage", "drainage (very important to soil health)", "well drained (prevents flooding and ponding)", "water infiltration", "tile"
pH	Soil	"ph", "ph 6.6 to 7", "proper pH", "soil pH", "pH level", "pH for crop being grown", "pH factor"
Cover Crops	Practice	"cover crops", "cover crops/no-till", "cover crops (good to practice)"
Crop Rotation	Practice	"crop rotation"
Chemical Inputs	Practice	"insecticides (GMO crops help us use less insecticide)", "chemical carry-over", "amount of weed killer chemicals (overuse)", "pesticides"
Soil Structure	Soil	"soil texture", "large air to soil particle size (warms up faster in spring)", "soil structure (this can have a positive or negative effect on soil health)", "stones and rocks probably was not overworked (usually has a history of being in pasture due to expensive repairs to farm mechanically, created better soil over time) ", "soil structure"
Fertilizer	Practice	"fertility fertilizer", "synthetic fertilizer (using some is beneficial, too much can be a negative) ", "amount of fertilizer needed to produce a profit and protect environment", "overuse amounts of nitrate fertilizers"
Disease & Pests	Soil	"insects", "bad bugs", "pests/disease", "disease carry-over", "soil borne pests (e.g. nematodes)"
Weather	External	"rain and sun", "weather"
Residue	Practice	"cover provided", "amount of compost (residue)", "crop residue"

Table 4.S1 (cont'd)

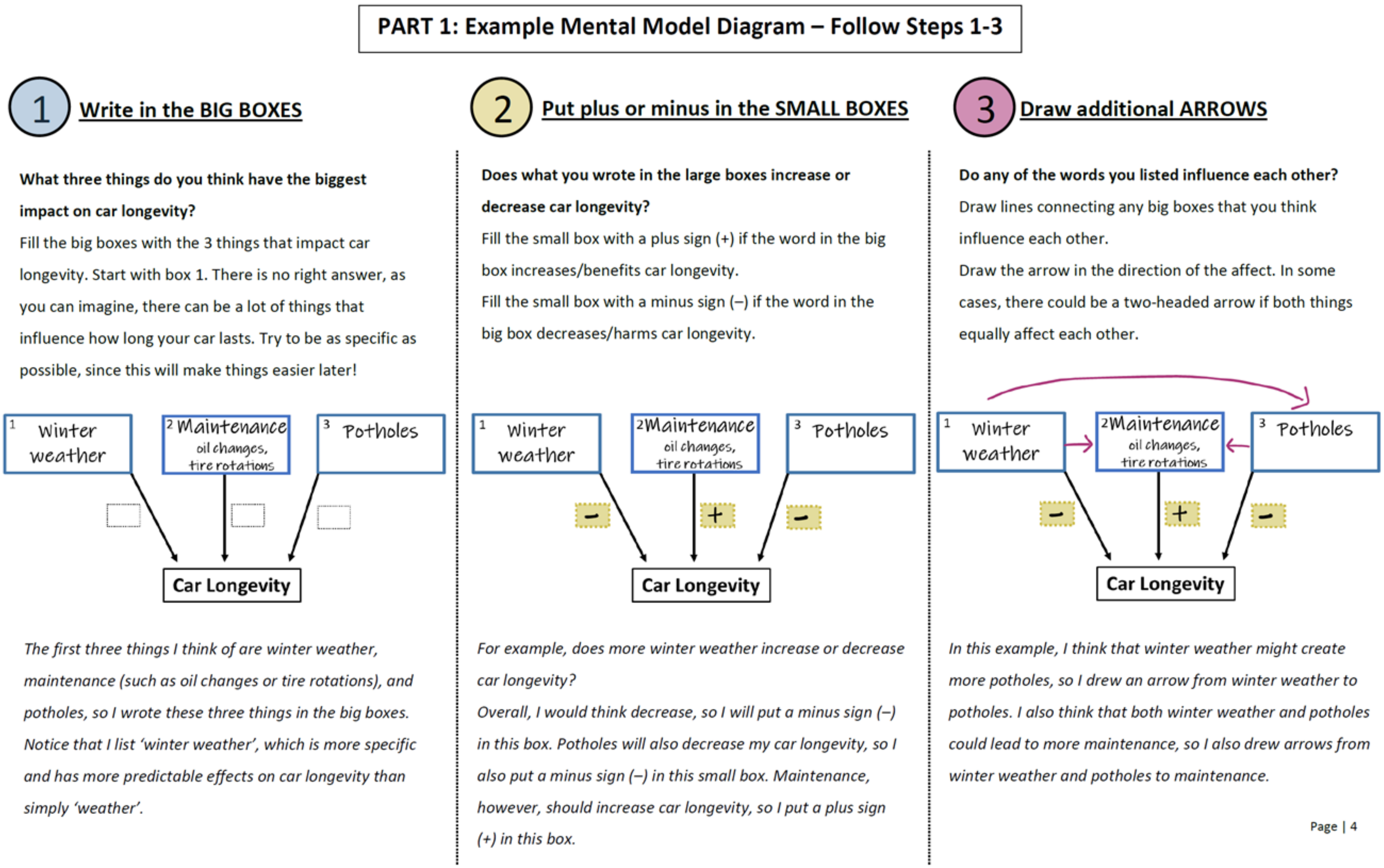
Topic	Category	Terms Aggregated
Production Practices	Practice	"producing in optimum conditions", "farming practices (probably too broad but I don't know how to split it up…)", "production practices", "management"
Water Holding Capacity	Soil	"water holding capacity", "water holding"
Erosion	Practice	"erosion (wind/water)", "reduced erosion", "erosion (top soil depth?)"
Soil Type	Soil	"top soil", "soil type", "type of soil", "sub-soil"
Weeds	External	"excessive weed pressure", "weed pressures"
Soil Moisture	Soil	"moisture held", "moisture (excessive or too little moisture is a negative, adequate is positive)"
Soil Tests	Practice	"soil testing", "soil sample"
Continuing Education	External	"continuing education"
Manure	Practice	"manure"
Soil Mapping	Practice	"soil mapping"
Overuse	Practice	"overuse"
Plant Diversity	Practice	"plant diversity (idle cropland? trees?)"
Irrigation	Practice	"water retention (irrigation?)"
Topography	External	"topography too hilly and uneven (soil erosion, hard to get soil and nutrients from washing away to another location)"
Bare Soil	Practice	"leaving bare soil exposed to the elements of weather"

Table 4.S2. Terms included in farmers' crop productivity mental models, which were aggregated at two-levels, by topic and category. Topics are sorted in order of frequency (see Figure 4.5).

Topic	Category	Aggregated Terms
Weather	External	"weather (can't control this very big factor)", "weather", "rain", "climate and weather (poor weather is a negative, good is a positive)", "storms", "Weather (rain/sun timing)", "water/weather", "good rain", "fall weather", "sunlight"
Disease & Pests	Soil	"disease", "Fungus pressure", "pest and diseases", "Insect Pressure", "pest pressure (insects, fungus, animals, etc.)", "fungus control", "pest control (insects, animals)", "insects", "pressure from herbicide and insecticide weeds and insects", "pests: insects, weeds, disease", "pests", "insect control", "disease/pests"
Seed Genetics	Crop	"seed attributes ", "seed", "seed/genetics", "seed selection", "seed quality", "seed quality and variety", "kind of seed (reg/gmo, day of maturity of crop)", "good seed", "crop tolerance to drought", "Hybrid Used", "good seed genetics"
Fertilizer	Practice	"fertilizer", "fertilizer program", "Fertilization", "manure", "sufficient added fertilizer/nutrients", "proper fertilizer rates and timing", "applied nutrients"
Weed Control	Practice	"weed control", "weed pressure", "weed resistance to herbicide"
Fertility	Soil	"soil fertility", "fertility", "good soil", "good soil fertility"
Drainage	Soil	"drainage", "drainage tile", "drainage (affects productivity greatly)", "good drainage"
Compaction	Soil	"compaction", "soil conditions (compaction)"
Timeliness	Practice	"timeliness", "timing of events (bad timing example: planting too wet, compacting soils, applying nutrients at the wrong time)", "timely harvest", "timeliness of harvest"
Planting Date	Crop	"plant date", "planting early", "drain well in the spring (early planting)", "planting date", "early planting"
Soil Moisture	Soil	"sufficient water", "adequate moisture", "moisture", "too wet", "droughty top soil (added manure helps)"
Soil Type	Soil	"soil type", "soil productivity (type)", "good subsoil composition (can't change)", "sand and silt or clay particles too high (not enough ions to attach nutrients) ", "type of soil"

Table 4.S2 (cont'd)

Topic	Category	Aggregated Terms
Pesticides	Practice	"herbicide application", "herbicides", "herbicide program", "pesticides"
Plant Population & Health	Crop	"planting effectiveness", "easy to make good seed to soil contact (better germination, more even stands)", "plant health", "plant population"
Crop Rotation	Practice	"crop rotation", "rotation of crops"
Growing Degree Days	External	"growing degree days for season", "adequate heat units", "degree days", "heat units"
Input Costs & Availability	External	"availability of inputs", "input costs", "affordability of inputs"
Market	External	"marketing", "strong demand (global weather? global politics?)", "market prices", "Market prices"
Soil Health	Soil	"soil health"
Machinery	External	"equipment problems", "excessive and abrasive wear on machinery (too much sand) ", "machinery prep"
Irrigation	Practice	"irrigation"
Field Conditions	External	"field conditions", "planting and harvest conditions (soil type, wet spots tiled)"
Management	External	"management"
Field Obstacles	External	"infrastructure, roads, electric", "tree lines"
Labor	External	"labor, man hours", "availability of labor"
Tillage	Practice	"tillage practices", "proper tillage and planting"
pH	Soil	"soil pH", "correct ph"
Soil Texture	Soil	"stones and rocks (history of less abused tough to work, more skips when seeding)", "soil texture"
Organic Matter	Soil	"organic matter", "CEC levels and organic levels too low (less capacity to hold water, nutrients, air)"
Soil Tests	Practice	"soil test"
Regulation	External	"regulation (some regulations help others hurt, usually they hurt)"
Soil Condition	Soil	"soil condition"
Topography	Soil	"slope"
Crop Loss	Crop	"crop loss at harvest"
Soil Preparation	Practice	"soil preparation"



PART 1: Example Mental Model Diagram – Follow Steps 1-3

1 **Write in the BIG BOXES**

What three things do you think have the biggest impact on car longevity?

Fill the big boxes with the 3 things that impact car longevity. Start with box 1. There is no right answer, as you can imagine, there can be a lot of things that influence how long your car lasts. Try to be as specific as possible, since this will make things easier later!

The first three things I think of are winter weather, maintenance (such as oil changes or tire rotations), and potholes, so I wrote these three things in the big boxes. Notice that I list 'winter weather', which is more specific and has more predictable effects on car longevity than simply 'weather'.

2 **Put plus or minus in the SMALL BOXES**

Does what you wrote in the large boxes increase or decrease car longevity?

Fill the small box with a plus sign (+) if the word in the big box increases/benefits car longevity.

Fill the small box with a minus sign (–) if the word in the big box decreases/harms car longevity.

For example, does more winter weather increase or decrease car longevity?

Overall, I would think decrease, so I will put a minus sign (–) in this box. Potholes will also decrease my car longevity, so I also put a minus sign (–) in this small box. Maintenance, however, should increase car longevity, so I put a plus sign (+) in this box.

3 **Draw additional ARROWS**

Do any of the words you listed influence each other?

Draw lines connecting any big boxes that you think influence each other.

Draw the arrow in the direction of the affect. In some cases, there could be a two-headed arrow if both things equally affect each other.

In this example, I think that winter weather might create more potholes, so I drew an arrow from winter weather to potholes. I also think that both winter weather and potholes could lead to more maintenance, so I also drew arrows from winter weather and potholes to maintenance.

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Figure 4.S1. Mental model example provided in farmers' activity workbook. Participants were asked to read this example before depicting their own mental models for crop productivity and soil health.

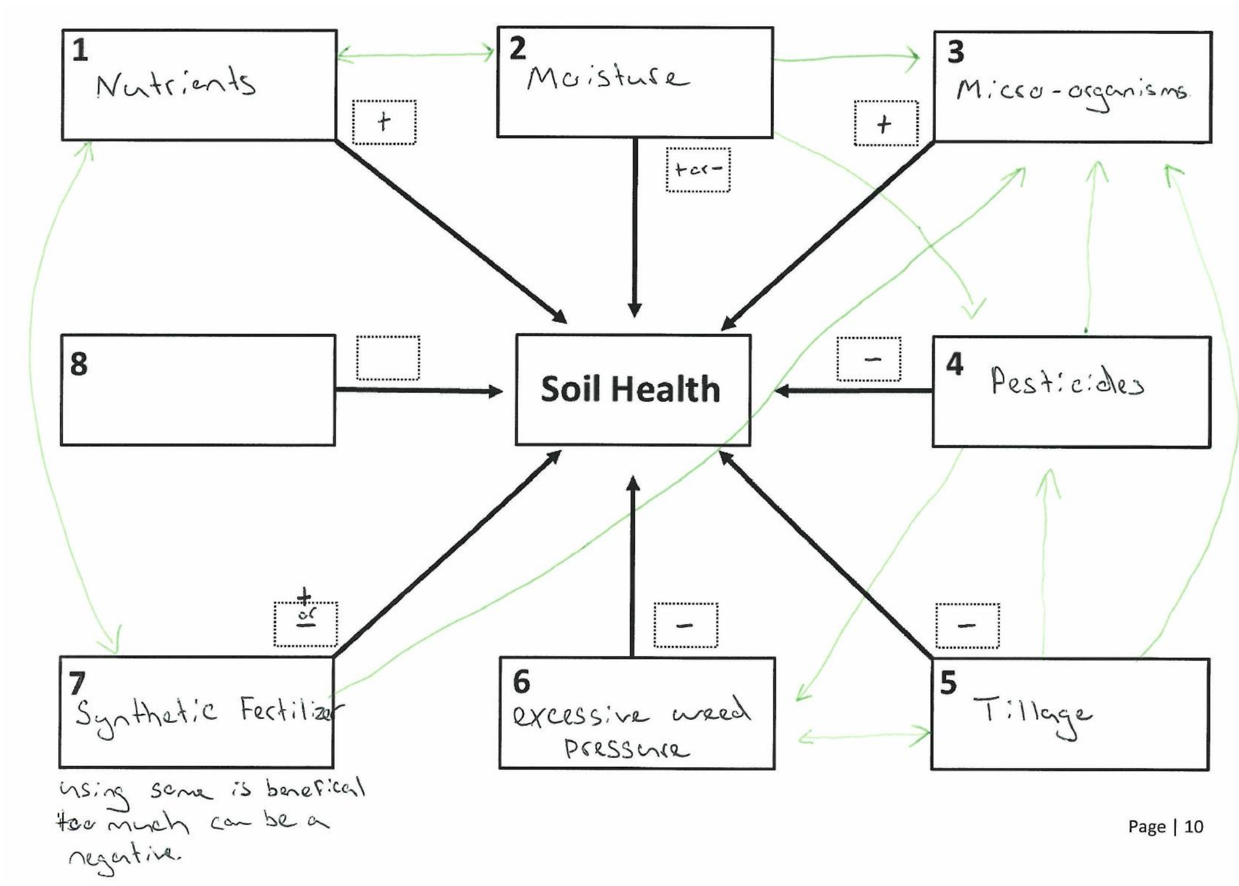

Figure 4.S2. Soil health mental model diagram that was mailed to all interview participants (n = 20). The farmers were instructed to 1) write in the big boxes to answer "what eight things do you think have the largest effect on soil health on your farm?", 2) use plus or minus signs in the small boxes to answer "does what you wrote in the large boxes increase or decrease soil health?" and 3) draw additional arrows between the factors to answer "do any of the words you listed influence one another?" This activity was first done for the crop productivity mental model and then the soil health mental model. Only the factors in the big boxes, not the plus/minus signs or arrows, were used for the analyses in this manuscript.

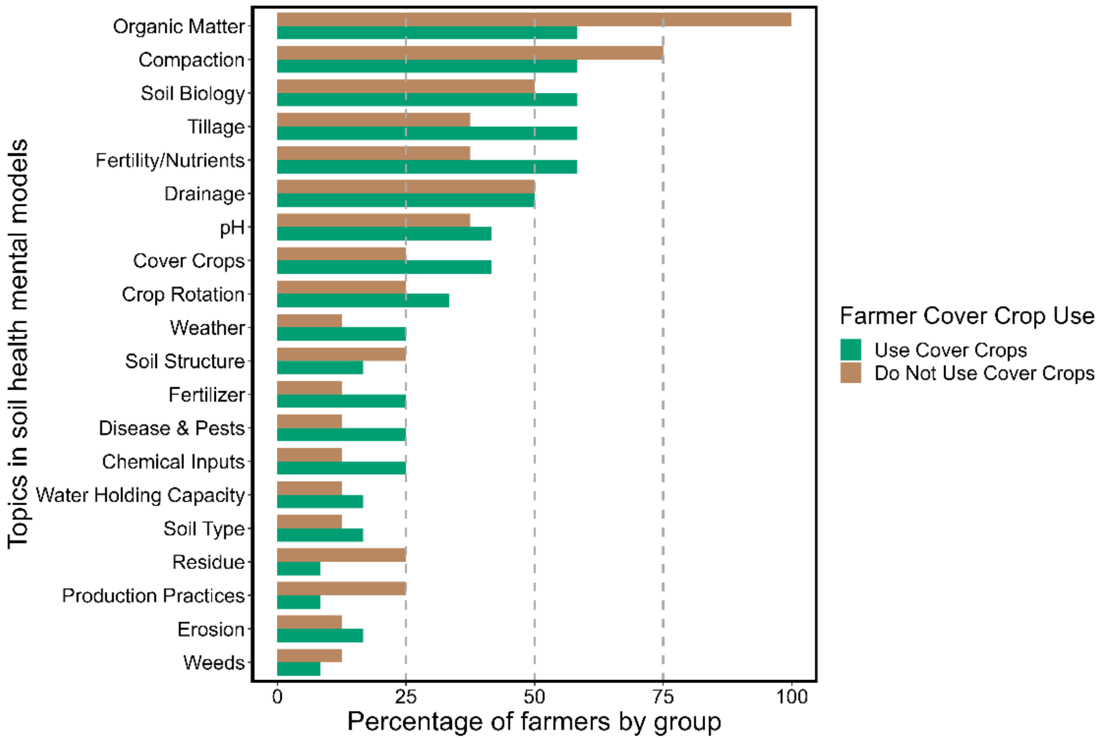

Figure 4.S3. Topics included in Michigan farmers' soil health mental models spilt by farmers' cover crop use (green: n = 12 use cover crops, brown: n = 8 do not use cover crops). Each topic consisted of several terms aggregated (Table 4.S1). Figure only includes topics that were mentioned at least one time by either group. Gray-dashed lines indicate 25%, 50%, and 75% of farmers.