

Studying the Plant-Microbe Interface of *Populus*
Using Constructed Microbial Communities

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ABSTRACT

The association of plant and microbes at the root-soil interface exemplifies a complex, multi-organism system that is shaped by the participating organisms and environmental forces. The plant microbe interface is a dynamic boundary across which a plant detects, interacts with, and may alter its associated biotic environment in order to maintain or improve its performance. Poor understanding of the mechanics of the plant-microbe interface represents a critical knowledge gap. Our goal was to investigate key areas of this gap: (a) microbial community assembly dynamics on *Populus* host root systems, (b) potential host specificity of two *Populus* species, and (c) the effect of environmental factors in structuring the root microbiome of *Populus*. This study used constructed communities in which specific microbes are combined with an axenic host in a controlled fashion. The process used two communities of 10 bacterial strains isolated from two poplar species; the 10 bacterial strains represented abundant members both functionally and phylogenetically from *Populus* natural microbiomes. The two communities were inoculated onto two *Populus* host species, and microbial community structure and abundance was assayed by qPCR and/or 16S rRNA amplicon sequencing. A time course study revealed that *Pantoea* dominates the community at all sampled time points and *Paraburkholderia* emerges as a dominant member as time progresses. In addition, species of *Populus* were dominated by *Paraburkholderia* and *Pantoea* or *Rahnella* strains regardless of original host species isolated from. Community members colonized in similar abundances compared to colonization by individual members of the communities. The shade treatment had no effect on the structure of the bacterial community, although

stem length and root area of the plant increased significantly with the 10-member community. This study demonstrates the feasibility and analysis of model communities to study microbiome function in plant systems.

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LIST OF ABBREVIATIONS

IAA: Indole Acetic Acid

NGS: Next Generation Sequencing

PCR: Polymerase Chain Reaction

qPCR: Quantitative Polymerase Chain reaction

PSR: Phosphate Starvation Response

QIIME: Quantitative Insights into Microbial Ecology

CFU: Colony forming Unit

CHAPTER 1

INTRODUCTION

Feeding mankind relies on high and stable yields from efficient crop production. Modern agriculture is mainly based on the cultivation of high-yield varieties combined with use of agrochemicals, i.e. fertilizers and chemical products, for nutrient input and pathogen control, respectively. Mineral fertilizers are derived from finite sources and agrochemicals are often hazardous to the environment, and as a result there is demand for an alternative and more sustainable agricultural practices¹. Microorganisms can affect agricultural productivity, for instance, by assisting and controlling nutrient availability/acquisition and promoting stress tolerance to the plant host. Considering the microorganisms as an active component of the host also responsive to changes in environmental conditions, it is imperative to gain a better understanding of the most important drivers of the composition and functioning of plant microbiomes.

Surveying the Plant Root Microbiome

A microbiome is a community of microorganisms including, bacteria, archaea, and fungi, as well as viruses that occupy an ecosystem or organism. A plant host provides a multitude of ecological niches for microorganisms to grow and subsist, allowing diverse microbes to coexist as a community². Plants host distinct microbes either on or near their root surface (rhizosphere), leaf surface (phyllosphere), or inside their tissues (endosphere) designated as the plant microbiome³⁻⁵. Reference in this text to *microbes* or *microbiome* refer exclusively to bacterial communities. The bacterial species within these communities vary according to the niches they occupy and the effects of different

microenvironments⁶. Some of the major drivers of these microenvironments are resource gradients such as nutrients, pH, physical space, reducing agents, and terminal electron acceptors⁶. Plants display a diverse array of interactions with these microbes, such as mutualistic, commensal, neutral and pathogenic⁷. The plant microbiome has been shown to benefit the host by providing nutrients through phosphorus solubilization and nitrogen fixation, the ability to synthesize plant hormones such as indole-3-acetic acid (IAA), and by secreting siderophores⁸⁻⁹. IAA increases the number of lateral and adventitious roots, enabling access to nutrients and enhancing root exudation, thereby offering more resources for microbes to interact with roots¹⁰. Secreted siderophores are organic compounds that enable chelation of iron for microbial and plant cell uptake in iron limited conditions¹¹. Similarly, phosphorus solubilizing bacteria can solubilize immobile phosphorus in soil that is available for plants to absorb for growth¹². On the other hand, due to their sessile lifestyle, plants are continuously challenged by biotic and abiotic stresses. Plant-associated microbes have the capacity to promote protection of the plant by hindering agents of plant stresses, including infection of pathogens and pests¹³⁻¹⁵, and abiotic stresses such as salinity, heat, and drought¹⁶.

Soil is a rich and diverse microbial reservoir that acts as source for plant microbiomes¹⁷. Soil types and soil characteristic can influence the microbes that interact with the plant host. Different types of soil can harbor diverse microbial communities¹⁸. The physico-chemical properties of soil (pH, structure, texture, organic matter, availability of nutrients) can directly select for specific microbes by creating niche environments that benefit certain types of microbes and influence the availability of plant

root exudates, thus affecting microbial recruitment by the plant¹⁹. The rhizosphere, defined as the volume of soil influenced by roots, is not a region of definite size or shape but instead consists of a gradient in chemical, biological, and physical properties that change both radially and longitudinally along the root²⁰. Rhizosphere environments are rich in compounds secreted by both plants and microorganisms. These compounds released by plant roots are collectively known as rhizodeposits. They include simple sugars, complex polysaccharides, amino acids, proteins, a multitude of secondary metabolites, flavonoids, and remnants of dead and lysed root-cap and border cells²¹. There are distinct microbes that have been identified as thriving in the rhizosphere. This region of soil surrounding roots harbors a tremendous diversity of microorganisms, either free-living or intricately linked to their plant hosts²². The increased microbial number and activity in the rhizosphere compared to those in bulk soil are mainly due to the release of organic carbon by the plant roots²³. A substantial fraction of net carbon assimilation goes into the soil as rhizodeposition. Estimates of how much carbon is allocated to rhizodeposition vary widely among plant species according to plant age, soil type, and nutrient availability, but are on average 11%-17% of net fixed carbon²⁴. The surplus of easily available carbon makes the rhizosphere environment substantially different from that of the root-free zones. As a result, a significant effect on the composition of rhizosphere communities has been assigned to soil types and plant species suggesting a contribution of soil and plant species on microbial communities²⁵⁻²⁶. Thus the rhizosphere environment plays a key role in maintaining plant-microbe interactions²¹. Recent studies have emphasized the significance of hormones involved in plant immunity and,

particularly the role of salicylic acid, in influencing the root microbiome²⁷. Studies with *Arabidopsis*, barley, maize, potato and sugarcane revealed a genotype-dependent variation in the composition of the rhizosphere community in addition to a soil-dependent variation^{2, 27-28}.

Furthermore, all plants organs have been found to host microbial communities, designated as endophytes, in their internal tissues without causing any host damage. Endophytes can colonize in the stem, roots, petioles, leaf segments, fruit, buds, seeds, and the dead and hollow hyaline cells of plants²⁹⁻³⁰. Root exudates, such as organic acids, amino acids, and proteins, may be involved in recruiting endophytes from the rhizosphere³¹. These microbes are able to overcome the host defense response and establish themselves as inhabitants of internal tissues without causing harm to the host plant, thus providing them with a protected environment with presumably less competition for nutrients³²⁻³³. The population of endophytes in a plant species is highly variable and is dictated by components such as soil types, host species, host developmental stages, and environmental conditions^{31, 34}.

In recent years, with the advent of Next Generation Sequencing(NGS) and state of the art bioinformatic software and workflows, microbial inhabitants of roots have been identified through culture-independent approaches, such as Polymerase chain reaction (PCR) amplification sequencing of the 16S rRNA genes, or through shotgun whole genome sequencing approaches³⁵. Genomic surveys have revealed that a single root can host a staggering taxonomic diversity of bacteria. Studies of plant root microbiome across different plants have shown remarkably similar distributions of microbial phyla;

invariably, members of the phyla *Actinobacteria*, *Bacteroidetes* and *Proteobacteria*, and *Firmicutes* were enriched ³⁶.

Plant root microbiomes represent an enticing example of interactive processes between microbes-host and microbe-microbe interaction that vary with ecological scale. Moreover, gaining mechanistic insight into the formation and function of host-associated microbial communities is important as the associated host microbiota contributes to a host's phenotype and growth. A structuring principle that governs the architecture of diverse community configuration must exist; however, this principle has not yet been adequately described. As these associations affect reproductive fitness, it is important to understand the functions of microbiota in terms of host fitness and how they are accomplished. The outcome of these interactions has profound consequences for modulating key ecosystem process soil nutrient cycling and other important ecological processes that are concurrently linked.

***Populus* as a Model to Study Plant Microbiome Interaction**

Populus has become the model woody perennial organism for researchers interested in testing mechanistic hypotheses related to plant–microbe interactions³⁷; *Populus trichocarpa* was the first tree species to have its genome sequenced³⁸⁻⁴⁰. *Populus* is a good choice for experimentation due to its fast growth rate and ability to propagate vegetatively³⁸. Vegetative propagation of poplar forces it to bypass the immature seedling phase and reach maturity sooner; moreover, the plant with desirable traits can be produced indefinitely⁴¹. Distinct microbiome composition of the *Populus* rhizosphere and root endosphere has been demonstrated across environmental gradients and between

Populus genotypes or species³⁹. Gottell et al., found that rhizosphere habitat was dominated by *Acidobacteria* and *Alphaproteobacteria*, while most endophytes were comprised of *Gammaproteobacteria* as well as *Alphaproteobacteria*³⁹. Microbial community isolates from *Populus* have also been shown to enhance the health, growth, and development of plant hosts⁴²⁻⁴³. Most of the studies done so far have focused on *Arabidopsis* – maize and rice – and these plants have a very short life span; on the other hand, *Populus* is a perennial plant that grows for ages⁴⁴. *Populus* trees currently are cultivated for pulp and paper production and have potential as a cellulose-derived biofuel feedstock, which means that understanding these interactions may be particularly important socioeconomically. Focusing on a genome sequenced model will allow for consolidation of information on perennial woody plants, and the knowledge generated by studies of *Populus* can be applied to other systems for better plant productivity.

The Need for Plant Microbe Interaction Research

Microorganisms are ubiquitous and important to life on Earth⁴⁵. Their genetic and physiological diversity results in enormous metabolic potential. Most of these processes are accomplished by the joint effort of microorganisms with different functional roles. Researchers working with microorganisms are increasingly acknowledging the impact of these microbial inhabitants on their hosts.

Comprehending the factors that shape and influence these microbial ecosystems is essential from microbiological, ecological, and biotechnological points of view. There is a pressing need to integrate our understanding of both plants and their associated microbiota. Ensuring the sustainability of agriculture becomes more important in light of

the future challenges posed by climate change and the rapid growth of the human population⁵. Taking advantage of the microbiota at work, i.e., capitalizing the microbial traits that are beneficial to the host or environment or both, presents a promising avenue for the development of more sustainable next-generation agriculture. Translating basic plant-microbiome research into practice, as in the example of exploiting microbial traits to optimize plant growth, is one way to reveal new vistas for development.

CHAPTER 2

LITERATURE REVIEW ON CONSTRUCTED COMMUNITIES

The Need for Model or Constructed Communities to Understand

Plant-Microbe Interactions

Considering the limited taxonomy of plant associated microbes when compared to the vast diversity of soil microorganisms, suggests that plants occupy a highly selective microbial niche^{2, 26, 31}. One of the principal objectives of microbiome research is to learn the molecular basis by which host-microbe and microbe-microbe interactions sculpt and maintain microbial communities. Furthermore, research seeks to understand the role of individual microorganisms as well as their collective function in a community context. Natural microbial communities are diverse collections of microbes, many with unknown functions, which poses problems in delineating and scrutinizing molecular level interactions.

Even the simplest of the natural plant-microbial communities characterized to date contains thousands of species; it is usually not possible to experimentally identify which species in such characterizations are performing vital functions⁴⁶. Natural communities with their complexity present a significant impediment to answering the fundamental ecological questions pertaining to molecular and ecological bases of community level function and community properties (robustness structure, size, and diversity)⁴⁷⁻⁴⁹. On the contrary, most of the research on plant microbe interactions has focused on the functional roles of single microbial groups (specific species or organisms from the same genera) associated with plants⁵⁰⁻⁵². There is a growing awareness that the

monoculture of model microbes cannot fully answer the questions about the biology of multispecies microbial communities in nature⁵³⁻⁵⁶. An exploration of plant microbiomes that embodies the complete set of interacting microorganisms might lead to the portrayal of numerous other functions that the associated microbe exercises when interacting with a host plant^{4, 24, 27, 57}.

Recent advances in next generation sequencing technology have marked the beginning of a new era in gathering information on the genetic repertoire of microbial communities from various hosts. Studies using massive parallel sequencing have defined plant-associated microbial communities for a wide variety of plant species from as small as the model species *Arabidopsis thaliana* to as large as trees, even for crops like rice, lettuce, corn, and potato⁵⁸. While generating catalogs of sequencing data through various methods (e.g. 16S rRNA gene amplicon sequencing or whole genome shotgun metagenomics) is readily accomplished, interpreting the biological mechanisms involved in microbial communities and establishing causal relationships between the microbiota and plant phenotypes is still elusive. Addressing fundamental questions is challenging and necessitates developing a experimental systems that allow for reproducibility and modifications of selected biotic and abiotic factors in order to pinpoint and link changes at the genetic or molecular level to host and community phenotypes⁵⁹.

An alternative method for overcoming the limitation of studies on natural communities and one-on-one interactions is taking a reductionist approach and assembling constructed communities. Constructed communities are an assemblage designed by mixing selected strains using bottom-up combinations that mimic the natural

environmental features as closely as possible. These can act as model systems to assess the role of key ecological, structural, and functional features of communities that allow the dissection of the role played by distinct strains in complex interaction networks. The knowledge used to understand community ecology function can be translated into agricultural and industrial applications.

Assembling a community requires the culture collection of microorganisms. Culture collection can be derived from plants grown under different conditions (soil quality, different climates). The central goal of culture collection is to increase the representativeness and resemblance to the natural microbiota and facilitate community research (Fig. 1). Focusing on genome sequence model plants such as *Populus*, *A. thaliana* and others (rice maize, and soybean) has allowed for the consolidation of resources and information⁵⁸. A gnotobiotic system that refers to the environment for

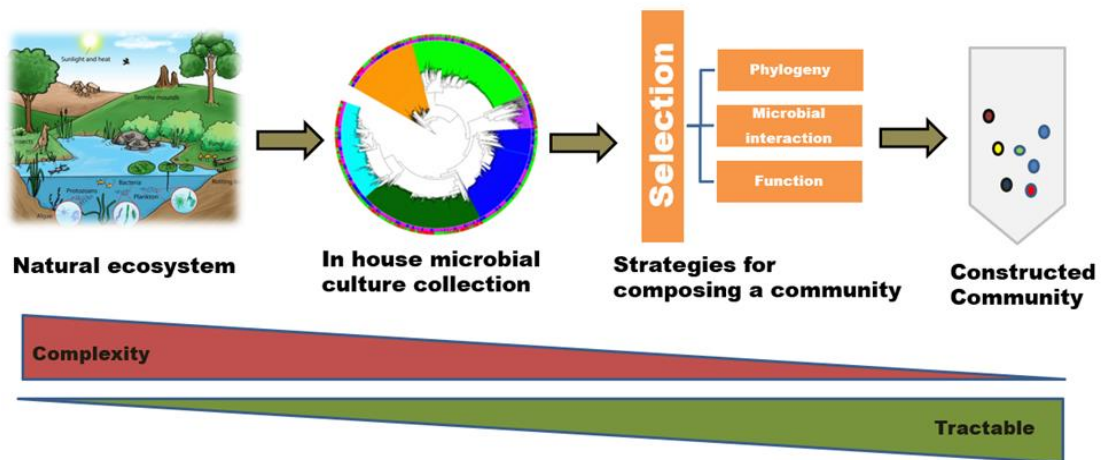


Figure 1. Framework for designing constructed microbial communities.

rearing or culturing organisms in which all the microorganisms are either known or excluded is ideal for asking questions regarding host dependent factors on specific elements of microbiome. Moreover, specific species and gene contribution to collective microbiome can also be studied. Additionally, variable environmental factors for plants include soil type, temperature, humidity, and intensity and quality of light, which are biotic and abiotic factors that can be manipulated to mimic a natural environment. Having maximum control over all these considerations can abate confounding factors and simplify the explanation of results. Proper control and targeted manipulation techniques can lessen the intrinsic complexity provided by genotype of the host, the genotype of the microbiome, and the environment in order to corroborate causality, preferably by integrating all layers that constitute the plant microbiome.

To appreciate the basic principles of community ecology and plant microbe interaction, it is important to assimilate copious data from careful experimental design and cutting edge sophisticated quantitative techniques. Understanding plant microbiota requires data with respect to relative and absolute abundance, spatial distribution, and molecular analysis. In the simplest form, cultivation dependent technique have been used to generate overall colony forming units from constructed community and to distinguish the individual strains based on selective media ⁶⁰⁻⁶¹. For more multifaceted communities, multiplexed amplicon sequencing on state-of-the-art sequencing platform that can handle hundreds of samples to generate relative OTU (operational taxonomic unit) abundances are in vogue ²⁷. PCR/qPCR approaches have also been applied to determine overall plant colonization of microbes ⁶⁰. To understand plant microbial interplay, genomic databases

of cultured collections have empowered the shift from phylogenetic to functional analysis. Proteomic methods have been employed in several studies to analyze microbial host adaptation in binary plant-microbe systems⁶². Metabolomics has been used to survey the metabolites on leaf surfaces as a function of colonization⁶³. Plant-microbe interaction research has been pursued with the intention of addressing the complexity of this interplay from the organism to molecular level.

Precedence of Using Constructed Communities in Literature

Research using constructed communities to investigate plant microbe interactions is in its embryonic stage, but these studies are witnessing rapid progress; there are several examples in recent literature in which constructed communities have been used. A simple constructed community of seven strains was assembled by Bodenhausen et al. to test how the composition of phyllosphere communities varies as a function of *A. thaliana* genotypes. This study demonstrated that a constructed microbial community established reproducibly on *Arabidopsis* leaves achieved steady state after relatively short time. Moreover, the screening of different plant genotypes (mutants in cuticle formation) displayed deviations in community composition and increased bacterial abundance relative to wild type plants. It offered a foundation for the fact that different bacteria can benefit from a modified cuticle to differing extents⁶⁰. Thus, this approach allowed for the establishment of a causal link between genotypes and phenotypes. Similarly, Lebeis et al. utilized a constructed community containing 38 bacterial strains to study the abilities of *A. thaliana* mutants with altered an immune system to shape the root microbiome. The authors examined mutants with deficiencies in biosynthesis and signaling of hormone

salicylic acid, jasmonic acid, and ethylene. They inferred that salicylic acid signaling affects the assembly of root microbiota. This study, carried out under more controlled conditions, allowed details at lower taxonomic levels²⁷; it also allowed for quantitative assessment of microbial characteristics with dynamic and spatial resolution by modifying host and environmental parameters. In another study, Bai et al., performed experiments directed to test varying microbiota composition on a single *A. thaliana* plant genotype. Competition experiments between leaf and root strains showed a competitive advantage during colonization of associated organ. Additionally, it was shown that, regardless of difference in initial inoculum of selected strains, the communities formed a stable community underlining its tendency to form a highly reproducible assembly⁶⁴. These communities are valuable because they are less complex than natural microbial communities and consequently experimentally controllable, which enables testing specific questions and hypotheses by controlled manipulation in gnotobiotic system.

Another study by Niu et al., utilized a representative seven species community of maize root microbiota, to demonstrate how this community assembles reproducibly and persists on the roots of axenic maize seedlings. Using a strain deletion experiment by eliminating one species at a time, the authors learned that the presence of *Enterobacter* species plays a key role for community assembly in an agar-based system. The absolute abundance of *Curtobacterium pusillum* escalated after removing *E. cloacae* from the system; on the contrary, other species were completely lost, signifying that the *Enterobacter* is the keystone species under the experimental conditions. Additionally, the study reported that the seven-member community was more effective at deterring the

causal agent of leaf blight disease, *Fusarium verticilloides*, than each member individually⁶¹. This study signifies that microbial assemblages can be propagated under carefully maintained experimental conditions. Experimental settings can be adapted to perturb, foster, or forbid precise types of microbial interactions. The growth of each microbial genotype can be measured in isolation, and in some cases within the assemblages themselves.

There have been studies by Castrillo et al., that described the assembly and effect of the root microbiota during plant nutrient stress. It was detected that by using a *A. thaliana* mutant with altered Phosphate Starvation Response (PSR) a different root microbiota was assembled both in soil as well as in agar system with a 35- member constructed community. Furthermore, the study reported, that the 35-member community enhanced the activity of transcriptional regulators and activated PSR under phosphate limiting conditions but also suppressed the activation of plant nutrition and immunity. Thus, by using a constructed community this study identified a molecular link between plant nutrition and immunity⁵⁷. The consequences of distresses (biotic or abiotic parameters) can be detected and investigated at different levels, which is essential for understanding the roles of individual microorganisms in community context.

Taken together, all the previous studies demonstrate the potential of constructed community studies as experimentally tractable, allowing modifications of microbial, host, and environmental parameters for the quantitative assessment of host and microbe characteristics with dynamic and spatial resolution. This approach thus enables testing of hypotheses to uncover plant microbe interactions.

The current study utilizes two communities of ten bacterial strains isolated from two *Populus* species (*P. deltoides* and *P. trichocarpa*). The ten strains represent abundant members both functionally and phylogenetically of the natural microbiome of *Populus*. The goal of this study was to investigate: (a) the community assembly dynamics on *Populus* host root systems, (b) the potential host specificity of two *Populus* species, and (c) the role of environmental factors in structuring the root microbiome *Populus*. The ten-member constructed community was assembled on the roots of axenic *Populus* plants grown in a magenta box system with sterile clay-based soil mimic. The two communities were inoculated into the soil, and rooted *Populus* cuttings were subsequently planted in the boxes; the microbial community structure and abundance were assayed by qPCR and/or 16S rRNA amplicon sequencing. The analysis of data for 16S rRNA amplicon sequencing was carried out with QIIME (Quantitative Insights into Microbial Ecology) pipeline⁶⁵. Additionally, the colonization of individual member on the roots of *Populus* was studied by a colony forming unit (CFU) counting on R2A agar plates.

CHAPTER 3

DEVELOPING AND TESTING OF MODEL CONSTRUCTED BACTERIAL COMMUNITY SYSTEM TO INVESTIGATE *POPULUS*- MICROBIOME INTERACTION

The microbiome of plants is comprised of thousands of taxonomically and functionally diverse microbiota, the majority of which are uncultivable⁶⁶. The association of plant and microbes at the root-soil interface exemplifies a complex, multi-organism system that is shaped by the participating organisms and environmental forces⁶⁷⁻⁶⁹. The plant microbe interface is a dynamic boundary across which a plant detects, interacts with, and may alter its associated biotic environment in order to maintain or improve its performance. Inadequate mechanistic understanding the plant-microbe interface represents a critical knowledge gap. This study presents the design of a model community of cultivable, genome-sequenced representatives of taxa in the microbiome of *Populus* plants and its applicability to study the plant-microbe interaction. The goal of this study was to investigate three areas of that interaction: (a) the microbial community assembly dynamics on *Populus* host root systems, (b) the potential host specificity of two *Populus* species, and (c) the effect of environmental factors in structuring the root microbiome of *Populus*.

Material and Methods

Bacterial strains and culture conditions

Bacteria were isolated using a dilution plating approach with three rounds of colony-restreaking on rich medium (R2A, Franklin Lakes, BD Difco, NJ, USA) agar

plates from either rhizosphere soil or surface-sterilized roots obtained from *Populus deltoides* plants growing near the Caney Fork River in central Tennessee, Yadkin river in western North Carolina or *Populus trichocarpa* growing in common garden sites in Oregon, USA^{39, 70}. Genomes of a subset of bacterial isolates were sequenced and assembled at Oak Ridge National Laboratory⁷¹⁻⁷² or through the Department of Energy Joint Genome Institute and are available at IMG. Strains were maintained using R2A liquid or agar medium. To prepare for inoculation, strains were grown overnight in R2A medium at 25°C and 200 rpm shaking. Bacterial suspensions were washed twice with 10mM MgSO₄, then diluted to OD₆₀₀ = 0.01 for plant inoculation experiments.

Plant culture

Arabidopsis thaliana Col-0 seeds were surface sterilized, plated on agar plates composed of 1/2× MS salts (Caisson Laboratories, Logan, UT, USA) and 0.7% phytagar (Caisson Laboratories, Logan, UT, USA) 0.1% sucrose, stratified at 4°C for two days, and then moved to a growth chamber for germination. After four days, seedlings of equal size were transferred to fresh agar plates of the same media composition, and bacterial strains were streaked 1 cm below roots. Plates were incubated for seven days, and then plants were imaged using a Zeiss Axiovert stereo microscope (Carl Zeiss AG, Pleasanton, CA, USA) to measure main and lateral root lengths. This assay was repeated in biological duplicate, using 3–4 plants per plate (total $n = 12$ plants each).

Populus deltoides “WV94,” (ArborGen Inc. Ridgeville, SC, USA) or *Populus trichocarpa* “BESC819”) genotypes were utilized for microbial assembly experiments. For propagation of plants from stock tissue culture plants, shoot tips were harvested and

sterilized by washing for 5 minutes in 1% Tween 20, 1 min in 70% EtOH, and 12 min in 0.6% NaOCl, then rinsed for 5 minutes 3 times in sterile DI water. Cuttings were transferred to a tissue culture medium containing 1× the strength MS salts (Caisson Laboratories, Logan, UT, USA), 0.5% activated charcoal (Sigma-Aldrich, St. Louis, MO, USA), 2% sucrose, 0.05% MES (Sigma-Aldrich, St. Louis, MO, USA), 0.15% Gelrite (Plant Media, Dublin, OH, USA), and 0.1% PPM (Plant Cell Technology, Washington, DC, USA) and used in experiments or as stock plants for up to three rounds of sub-culture. Sub-cultured plants were grown in the same tissue culture medium described above for three weeks until rooted, then transplanted into experimental condition media.

Plant inoculation

Microcosms were constructed by interlocking two sterile Magenta boxes (SigmaAldrich, St. Louis, MO, USA), with 150 ml calcined clay (Pro's choice Sports Field Products, Chicago, IL, USA) and 70 ml of 1× Hoagland's nutrient solution (Sigma-Aldrich, St. Louis, MO, USA) added to each microcosm. Holes (7 mm) were drilled into adjacent sides of the upper magenta box and covered with adhesive microfiltration discs (Tissue Quick Plant Laboratories, Hampshire, United Kingdom) to allow air to flow into and out of the microcosms and to prevent outside microbial contamination. Prior to microbial addition, microcosms were sterilized by double autoclaving on a 60-minute dry cycle over consecutive days.

Bacterial strains were grown in isolation and at a constant temperature in 5 ml of R2A medium. After growing overnight, they were pelleted and re-suspended. Suspensions were washed twice with 10 mM MgSO₄, then diluted to an OD₆₀₀ of 0.01

($\sim 1.0^7$ cells/ml). The microcosm was inoculated by adding 10 ml of the bacterial strain (10^7 cells/ml) to the calcined clay substrate and stirring for 30 seconds to distribute the bacteria. *Populus* clones were planted within each microcosm after inoculation. Each *Populus* was grown in an individual microcosm in combination with constructed bacterial community.

Harvesting

Plants were grown in growth chambers under 16 hours of light, 8 hours of dark per day with $\sim 50\%$ humidity. Plants were harvested on days 1, 7, 14, and 21. Plants were harvested by carefully removing the entire plant from the soil, rinsing the root system in sterile DI water to remove loose soil, imaging and processing the tissue. Root material was flash-frozen immediately in liquid nitrogen and stored at -80°C .

Plant growth and physiology

Chlorophyll content was measured on fully expanded leaves with a SPAD-502Plus (Konica Minolta, Ramsey, NJ). Stem length was measured from the base of stem to the highest actively growing leaf.

CFU enumeration

Populus trichocarpa BESC819 were planted in a magenta box using similar methodology and treatment described above except that strains were inoculated separately. Plants were removed from the microcosm 21 days post-inoculation, submerged in sterile distilled water to remove the clay from the root system. The wet weight of plant root tissue was recorded, and one gram of root tissue was macerated with sterile pestle and mortar in 1 ml sterile MgSO_4 . Macerated plant tissue was subsequently

transferred to a 24-well plate and serially diluted with MgSO_4 at 1x, 0.1x, and 0.01x of the original sample concentration. Each sample was plated onto R2A medium plates and allowed to grow for 48 hours at 20°C, after which the colonies were counted. We calculated CFU/g of plant tissues by multiplying the colony number per plate by $10^{(\text{dilution factor} + 1)}$ then dividing that number by root tissue mass.

Sample processing, DNA extraction and quantification

Plant roots were the focal point of microbial community analysis. To homogenize the sample and disrupt plant cell walls, we flash-froze them in liquid nitrogen and homogenized them by bead-beating for one minute with a small steel bead (diameter=6mm). Next, samples were flash-frozen again for one minute so the sample would not thaw during the homogenization process. This process was repeated three times to ensure complete homogenization. DNA was extracted from homogenized tissue using PowerPlant Pro DNA Isolation kit (Qiagen, Germantown, Maryland, USA), with slight procedural modification for high concentration DNA yields. These modifications consisted of homogenizing in Precellys 24 (OMNI International, Kennesaw, Georgia USA) at 3200g for 3 minutes at 30-second intervals of pulse and rest. DNA was eluted in a 50 μl buffer. All extractions were quantified on a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA) and Qubit 2.0 (ThermoFisher Scientific).

16S library preparation and sequencing

The bacterial 16S rRNA gene was selectively amplified and barcoded by using established protocols utilizing Peptide Nucleic Blockers (PNA) to prevent plastid and mitochondrial 16S rRNA gene amplification⁷³. Libraries were prepped by means of a

two-step PCR approach with a mixture of 515F and 806R primer. An adapter sequence was added to each forward and reverse primer to make them compatible with Nextera XT indexes (Illumina). The initial polymerase chain reaction (PCR) consisted of 2× KAPA HiFi HotStart ReadyMix Taq (Roche, Indianapolis, Indiana, USA), 10 µmol/L total for each forward primer combination, and 10 µmol/L total for each reverse primer combination, with approximately 50 ng DNA. The first PCR reactions consisted of 3 minutes at 95°C, followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. Successful PCR amplification was confirmed by running 4 µL of PCR product on a 2% agarose gel. The PCR product was then purified by use of AMPure XP beads (Agencourt, Beverly, Massachusetts, USA). Nextera XT indexes were then ligated to the PCR products by use of a second, reduced cycle PCR so that each sample had a unique combination of forward and reverse indexes. This reduced reaction consisted of 3 minutes at 95°C, followed by eight cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. The products were purified again using AMPure XP beads. Samples were quantified on a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA), and Qubit 2.0 (ThermoFisher Scientific) and pooled to approximately equal concentration in each pool. Final product size and concentration were confirmed on a standard sensitivity Bioanalyzer (Agilent, Santa Clara, California, USA). Samples were diluted to 4 µmol/L, combined with 5% of a 4 µmol/L PhiX adapter-ligated library control, and run paired-end on a v2, 500 cycle flow cell of an Illumina MiSeq sequencer.

Sequence processing

Raw sequence reads were processed using cutadapt to remove primer sequences⁷⁴. Sequences were then imported into QIIME2⁶⁵ (v. 2018.11) for further processing. Sequence variants were assigned using dada2⁷⁵ implemented in QIIME2. Taxonomy was assigned to the sequence variants using a naïve Bayesian classifier trained on the SILVA 132 database⁷⁶. Sequence variants identified as chloroplast, mitochondria, or unassigned were removed from the further analysis. Taxonomy was also assigned using BLAST consensus implemented in QIIME2 against a database of 16S sequences from the bacterial community members used for inoculation with 97% nucleotide identity cutoff⁷⁷.

The taxonomy assigned using SILVA was used for sequence variants that did not match a member of the inoculum. The representative sequences for each sequence variant were aligned using MAFFT and filtered using MASK, both implemented in QIIME2. A phylogenetic tree was created using Fasttree from the alignment mentioned above⁷⁸. The resulting sequence variant table, tree, mapping file, and taxonomy file were imported into Phyloseq⁷⁹ (version 1.22.3) in R (version 3.4.4; R Core Team 2018) for further analysis⁸⁰.

Sequence analysis

Alpha diversity was measured using the Shannon diversity index. The statistical significance of alpha diversity measurements for inoculated and control samples was assessed using the Wilcoxon test with Benjamin Hochberg corrections implemented in the package ggpubr⁸¹ (v. 0.1.6.999). Sequence counts were rarefied to 100 sequences per sample prior to further analysis. Beta diversity was measured by creating a distance matrix on rarefied sequence counts using weighted UniFrac distances⁸², which is a

phylogenetic distance metric that takes into account sequence variant relative abundances. To test whether inoculation or days since inoculation had an effect on the bacterial communities, a PERMANOVA test was performed via the `adonis` function `vegan`⁸³(v.2.5-1) package on the weighted UniFrac distances. To test the effect of the number of days since the samples were inoculated on only the inoculated samples, control samples were removed and a PERMANOVA test was performed. All figures were created using `ggplot2` (v 3.0.0) in R

Results

Experiment I: Design of reference host-microbe community system based on sequenced isolates and functional characterization of selected strains with *A. thaliana* in plate assay to determine root phenotype

For the current study, bacterial species were selected from among a collection of >3000 isolates of *Populus* associated bacteria that is maintained as glycerol freezer stocks. Criteria for selection of bacterial strains included functional potential and phylogenetic diversity (Fig. 2). Two 10-member constructed communities consisting of isolates exclusively from *P. trichocarpa* or *P. deltoides* were designed (Fig. 3). Functional characterization of isolates derived from *P. deltoides* host was carried out with *A. thaliana* in plate assay to determine root phenotype (Fig. 4). Communities were selected to be phylogenetically similar, with all isolates in community Pd coming from *P. deltoides* host, and all isolates in community Pt coming from *P. trichocarpa*. Prior studies analyzing species composition of plant associated microbial communities were consulted to guide efforts in establishing a simplified root bacterial community^{39, 42-43, 84}.

Genus	Strain	Function of interest
<i>Streptomyces</i>	YR139	Diversity
<i>Rhizobium</i>	CF142	Quorum sensing, PipR, Salicin
<i>Sphingobium</i>	AP49	IAA production
<i>Caulobacter</i>	AP07	Phytate growth
<i>Bacillus</i>	BC15	AiiA Lactonase
<i>Burkholderia</i>	BT03	Plant growth promoting, Quorum sensing
<i>Variovorax</i>	CF313	Arbutin growth
<i>Duganella</i>	CF402	Aryl- β -glucosidase
<i>Pseudomonas</i>	GM17	Quorum sensing
<i>Pantoea</i>	YR343	Biofilm, c-di- GMP

Figure 2: Strains included in the community derived from *Populus deltoides* isolates for testing.

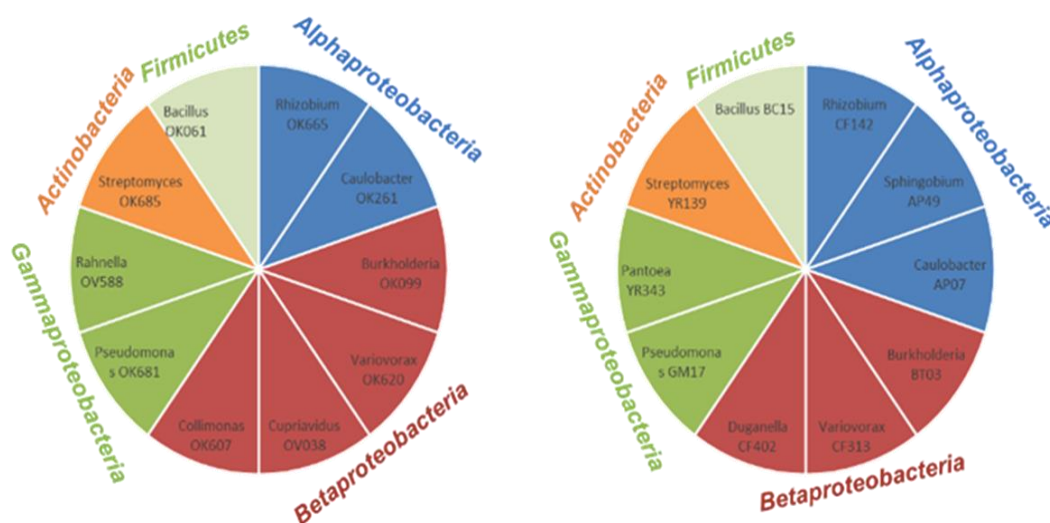


Figure 3: Selection of model communities. Community members were selected based on phyla level abundance to represent natural populus communities. On the left is community from *P. trichocarpa* isolates (Pt) On the right is community from *P. deltoides* isolates (Pd).

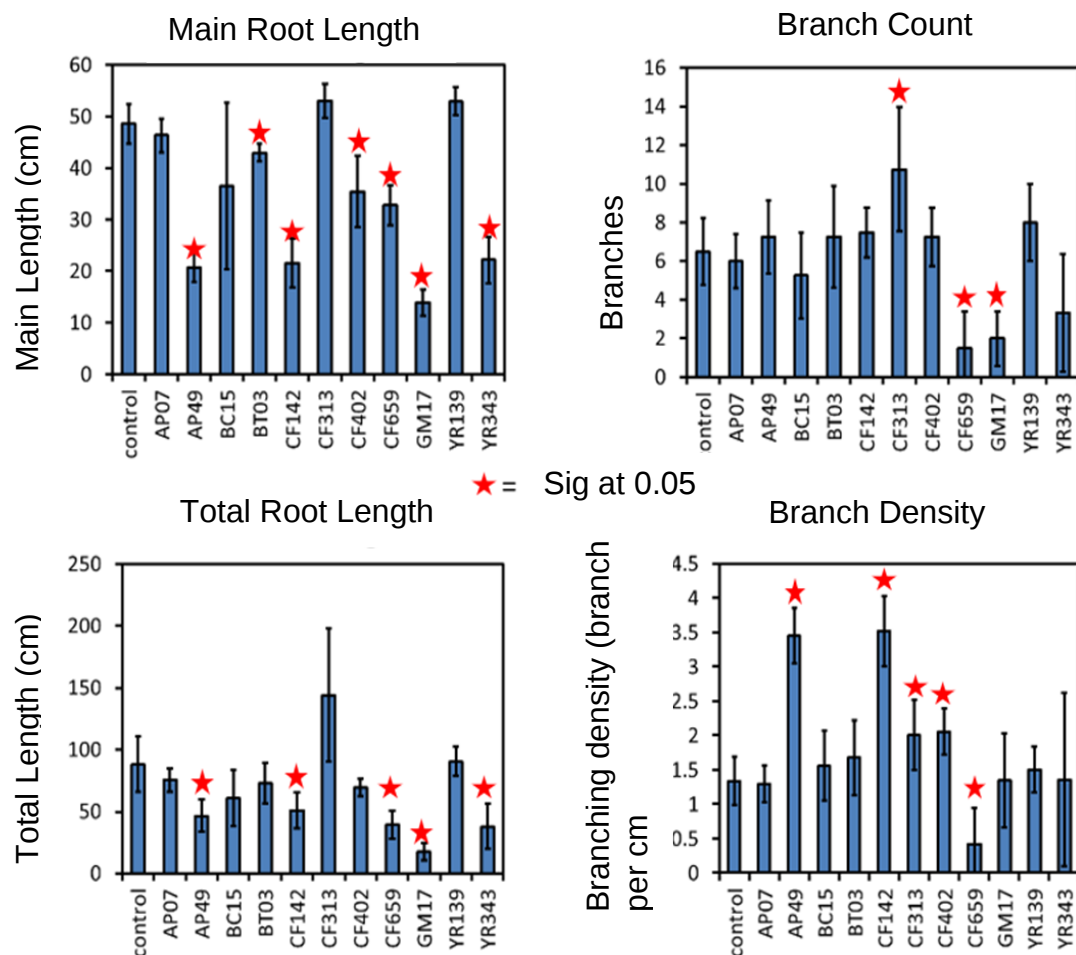


Figure 4: Functional characterization of bacterial strains. Strains were grown with *A. thaliana* in plate assays to determine root phenotype: Main root length, total length, branch count and branch density error bars are standard error for n=12 plants. All values are significantly different at $P < 0.05$ (Students' *t*-test)

Experiment II: The temporal dynamics and assembly of constructed community derived from *Populus deltoides* isolates on *Populus* roots determined over the course of 21 days

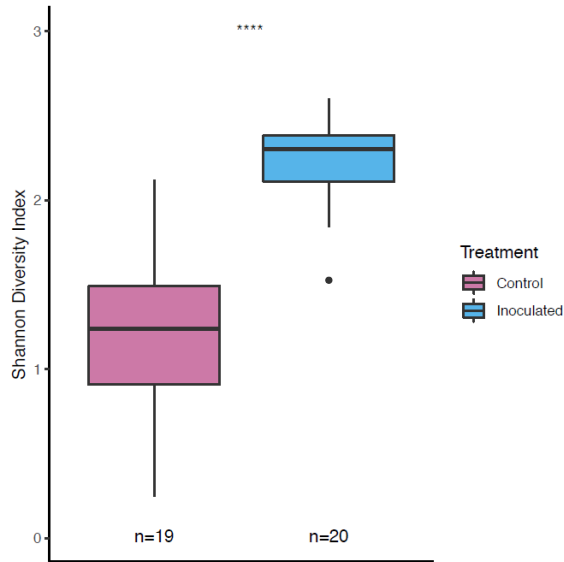
The aim of this study was to determine reproducibility and community assembly dynamics over the course of 21 days. The time course studies have the potential of increasing in depth analysis of microbiome over time that can provide insights into fundamental questions about microbiome dynamics. The study can determine the degree to which a bacterial community is deterministically assembled based on its initial composition and to what degree the microbial composition at a given time determines the microbial composition at later time.

Bacterial diversity and dynamics were determined by 16s rRNA sequencing as described above. Alpha diversity was significantly different between inoculated and control plants ($p=0.00000082$, Fig. 5a). When time was analyzed, alpha diversity did not significantly differ between control and inoculated plants at one day ($p=0.066$, Fig. 5b) but differed at all proceeding time points (7 days, $p=0.037$; 14 days, $p=0.012$; 21 days, $p=0.012$). Bacterial community structure was significantly different between inoculated and control plants, and inoculation explained roughly 50% of the variation between the communities ($r^2=0.506$, $p=9.9e-5$; Fig. 5c). Days since inoculation explained 36% of variation within the bacterial community ($r^2=0.364$, $p=0.002$; Fig 5d). Of the 10 bacterial strains used for inoculation, 9 were recovered with 16S amplicon sequencing (*Bacillus* sp. BC15 was not recovered). Uninoculated control plants were dominated almost exclusively by a *Microbacterium*, which most likely came along with *Populus* cuttings

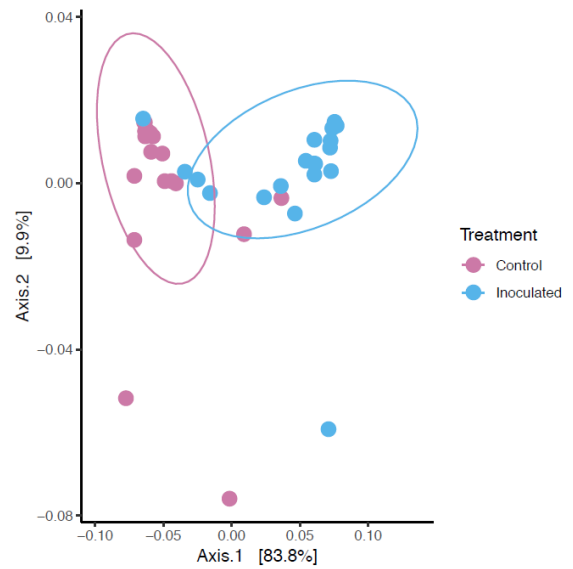
and relative abundance was independent from the days incubated. On the other hand, inoculated plants were also initially colonized by the *Microbacterium*, but as time progressed the inoculated strains became the major part of the community (Fig. 6). The *Pantoea* sp. YR343 and *Paraburkholderia* sp. BT03 strains made up more than 90% of communities in inoculated plants at the 21-day time point. *Pantoea* sp. YR343 appears to be an early colonizer and dominates the community irrespective of days post-inoculation, while *Paraburkholderia* emerges as the second dominant member as time progresses. Twenty-one days after the inoculation, a reproducible community assemblage across host replicates was observed.

Experiment III: Determining the potential host specificity of two *Populus* host species towards the constructed community

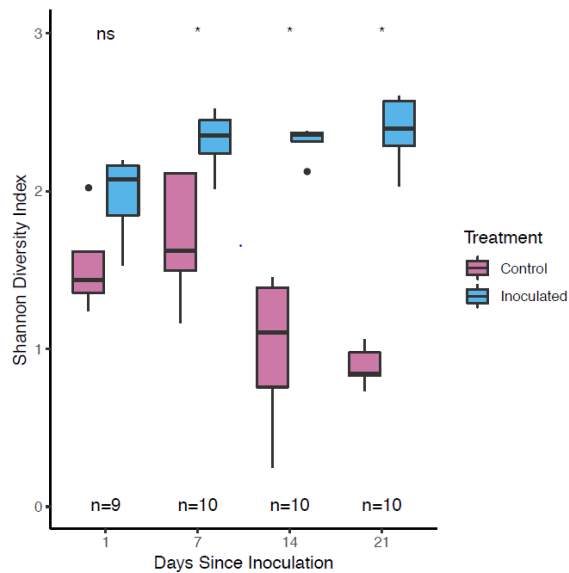
As a step after defining a constructed community that assembles reproducibly on the axenic *populus* roots, the two 10-member constructed communities were cross inoculated to study how the constructed community isolated from one *Populus* host species assembles on another *Populus* host species. For this, the community made from *P. trichocarpa* (Pt) was inoculated into the soil and rooted *P. deltoides* cutting were subsequently planted in the magenta boxes as described above. Similarly, the community made from *P. deltoides* isolates (Pd) were inoculated with the *P. trichocarpa* host roots (Fig. 7). Five replicates were used for each host treatment. The plants were incubated with inoculated community for 21 days, after which the roots were harvested, and further sample and sequence processing was carried out as described above.



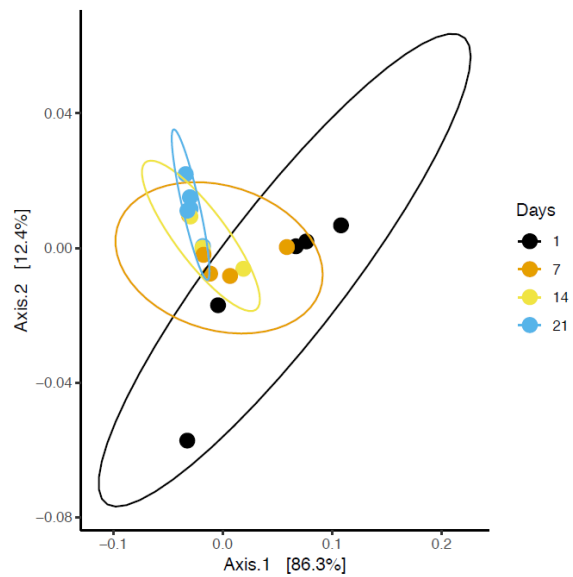
5a. Alpha diversity between inoculated and control plants.



5c. PCOA plot of inoculated and control plants.



5b. Alpha diversity between inoculated and control plants at each time point.



5d. PCOA plot of inoculated and control plants at each time point.

Figures 5a-d: Bacterial diversity measurements.

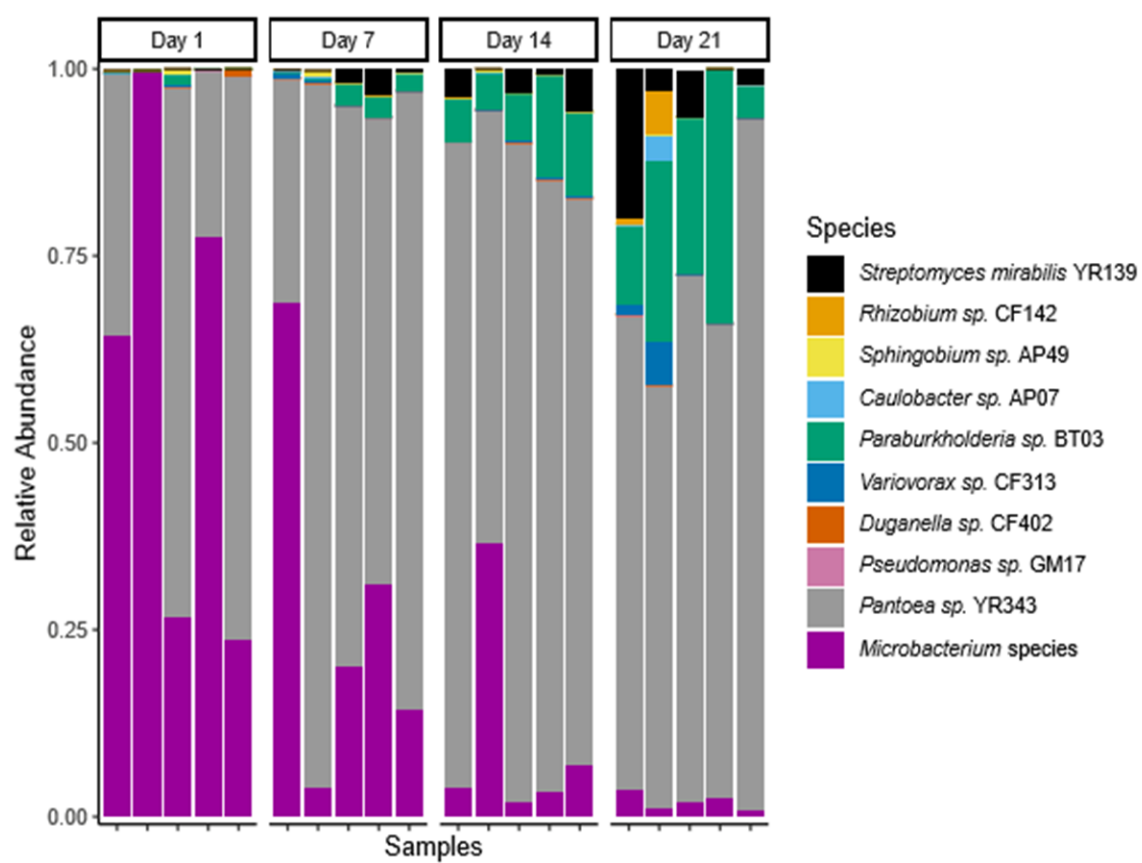


Figure 6: Histogram of the inoculated strain abundance at each time point per replicate.

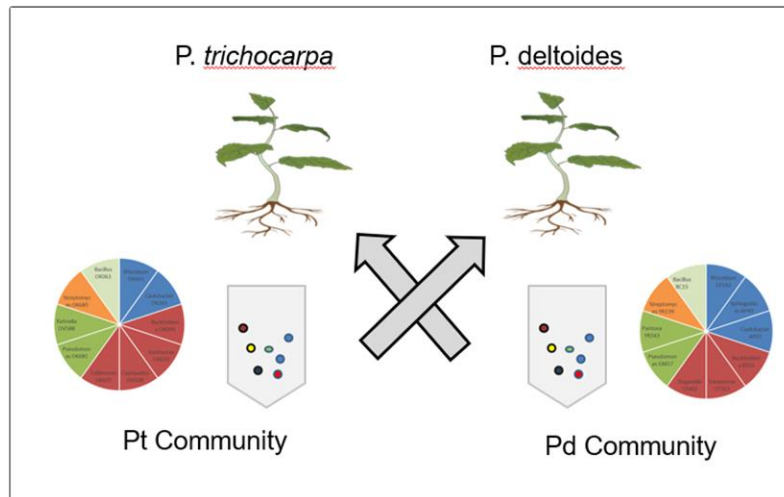


Figure 7: Cross inoculation of communities on *Populus* host genotypes.

Studies across 10 plants from each of two host species, indicated the community is dominated by two taxa, with *Paraburkholderia* and *Pantoea* isolates representing 99% of detected organisms. Within the remaining 1%, the *Streptomyces* and *Rhizobium* representatives are the most abundant, followed by *Sphingobium* and *Duganella* isolates. The community is dominated by *Paraburkholderia* sp. BT03 (formally *Burkholderia* sp. BT03) and *Pantoea* sp. YR343 in both the communities irrespective of the host species (Fig. 8). *Streptomyces* from the Pt community colonized poorly and was not represented in *P. trichocarpa* as well *P. deltoides* host. Similarly, *Bacillus* from Pd community isolate was not well represented on either of the host, this observation agrees with the previous experiment, using time course, where *Bacillus* sp. BC15 was not detected at 21 days. The 10-member community dynamics in this experiment mirrors the results from previous experiment already shown in the time course experiment, reinforcing that community assembly is reproducible across the two host species.

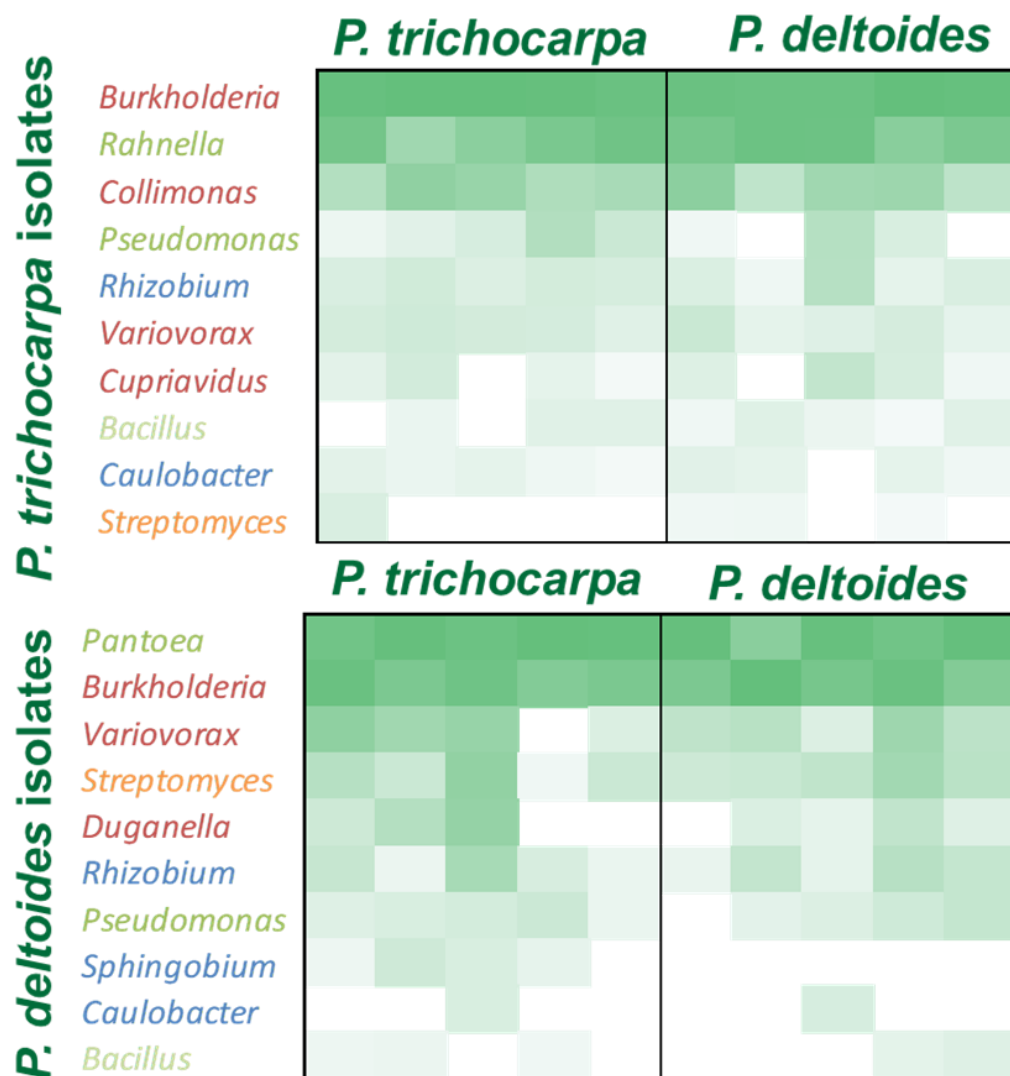


Figure 8: Relative abundance (16S) of constructed community. Color shade calculated by $\log_{10}$ transformation of relative abundance. Green means high abundance, white means low abundance.

Experiment IV: Determining the colonization of individual member of the constructed community on *Populus* roots

CFU enumeration data was compared against the qPCR data of Pd community on two hosts (Fig. 9). *Paraburkholderia* sp BT03 and *Pantoea* sp YR343 colonized the *Populus* roots significantly both individually and as part of a multi-member community. Strains *Calulobacter* sp. AP07 and *Bacillus* sp. BC15 that were not well represented in the mixed inoculation emerged were detectable when inoculated individually as quantified by cfu. These results suggest there is further interaction of the strains on hosts as well as with other members of their community.

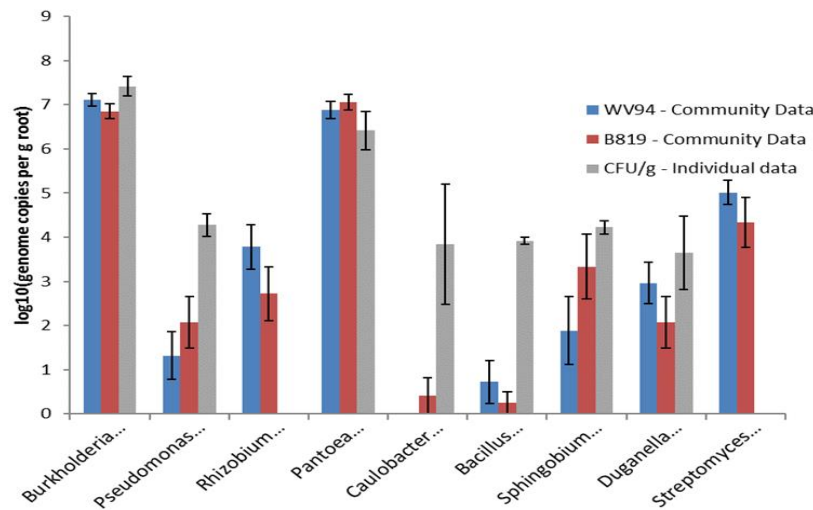


Figure 9: Average colony forming units of individual Pd community members across 10 replicates (grey bar). Compared against the qPCR data from community analysis (blue and red bar). Error bars in community data are standard error for 10 replicates per host. Error bars in Individual data are standard error for 3 replicates.

Experiment V: The effect of light treatment on constructed communities

Understanding how the plant and its associated microbiome respond to changes in the environment is critical for harnessing the protective and adaptive powers of the microbiome. Light availability greatly affects the plant growth and its productivity. Low light not only limits carbon availability for plant growth but also restricts the energy supply for essential metabolic processes⁸⁵. Shading and the ultimate effect on plant photosynthesis and carbon allocation shifts the association of the plant with beneficial microbes in the environment⁸⁶. Shading and cloud cover are natural limitations on light that decrease the overall biomass production and lead to structural changes in *Populus*⁸⁷.

This study sought to determine the response of plants subjected to shade, and the response that is reflected in the inoculated constructed community (It was hypothesized that an inoculated constructed community would mirror the host response to stress due to altered carbon allocation, showing a treatment-specific response in microbial abundance changes. Briefly, communities made from both *Populus trichocarpa* (Pt) and *Populus deltoides* (Pd) were inoculated into the soil and rooted *Populus trichocarpa* (BESC819), and cuttings were subsequently planted in the boxes as described above. Control plants were exposed to full light, while the experimental plants were subjected to shade (80%) by obstructing the light reaching the plants using meshed cloth (Fig. 10). A total of 6 plants were used for each set of control and treatment. Plants were incubated for 21 days, and roots were harvested and processed as described above.

Expt. Design	Shade	Control	
ctrl (no bacteria)	<i>n</i> =6 plants	<i>n</i> =6 plants	
<i>P. deltoides</i> isolates	<i>n</i> =6 plants	<i>n</i> =6 plants	
<i>P. trichocarpa</i> isolates	<i>n</i> =6 plants	<i>n</i> =6 plants	

Figure 10: Experimental design for light perturbation study.

The community composition in the root was measured by the 16S amplicon sequencing as described above. It was observed that, similar to previous experiments, *Paraburkholderia* sp. BT03 from *P. trichocarpa* isolates and *Pantoea* sp. YR343 from *P. deltiodes* dominated (Fig. 11). Shade treatment did not result in significant shifts in communities relative to the control, and treatment did not result in different communities relative to each other.

To determine how the host inoculated with constructed community is affected when subjected to shade, phenotypes were assayed for chlorophyll content and stem length. Chlorophyll measurement was measured on expanded leaves by Soil-Plant Analyses Development unit (SPAD-502 Plus, Konica Minolta, Ramsey, NJ). SPAD is nondestructive and non-invasive instrument to measures “greenness” or chlorophyll content of the plant instantly.

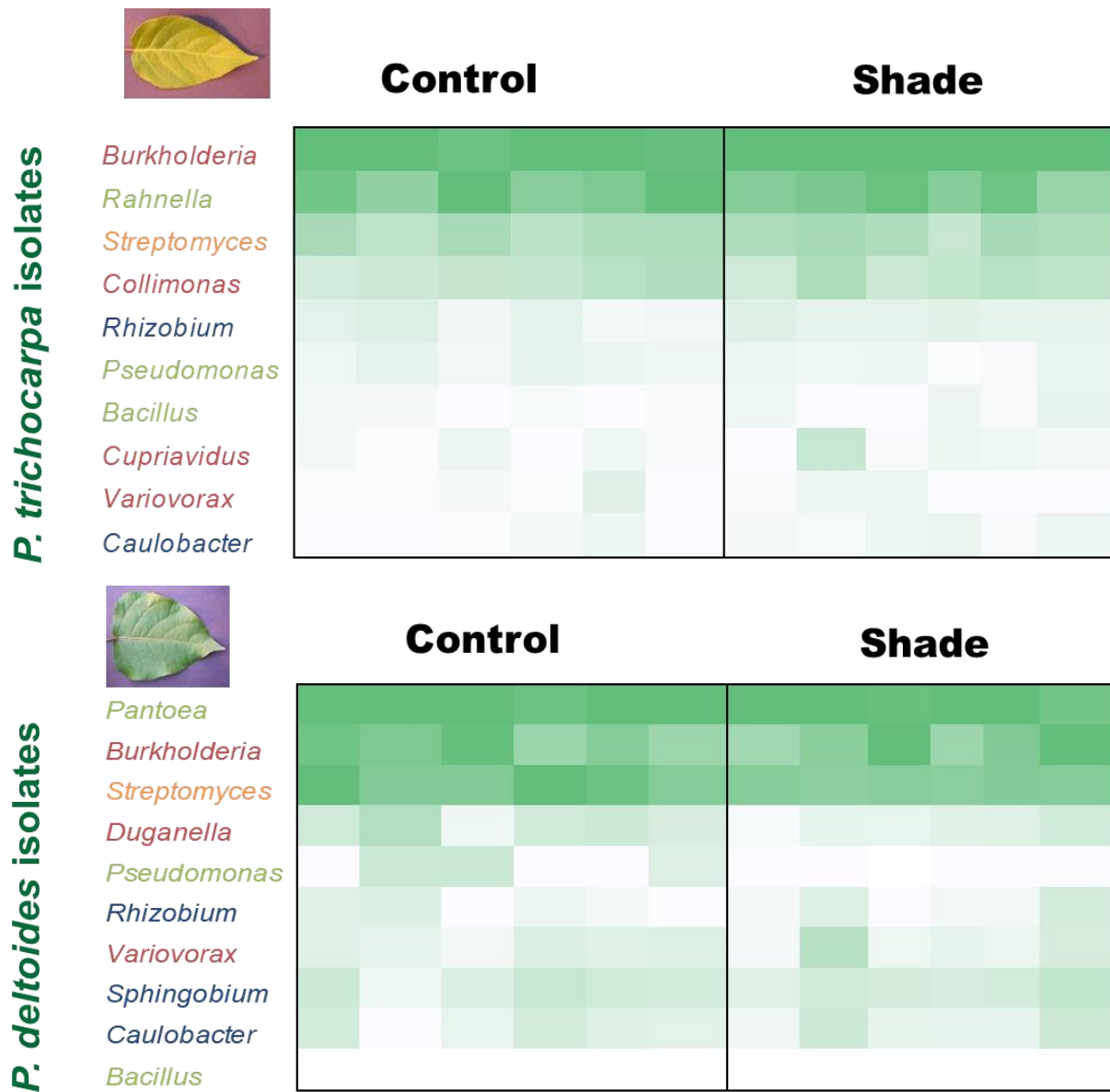
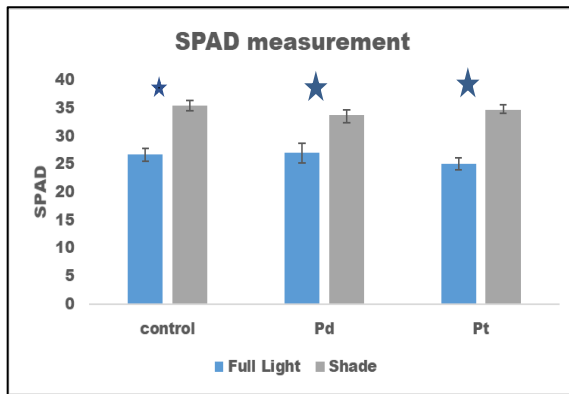
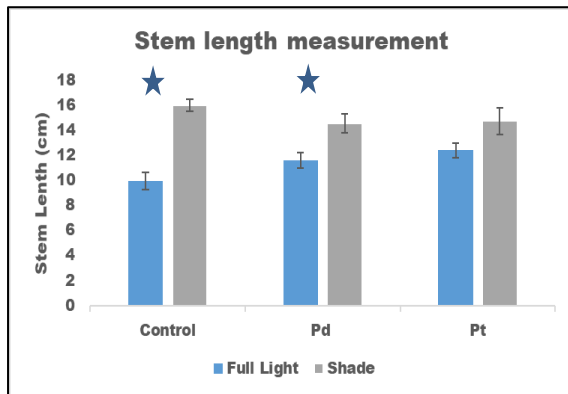


Figure 11: Relative abundance of constructed community strains under control and shade treatment. Color shades are calculated by Log_{10} transformation of relative abundance. Green means high abundance, white means low abundance.

With ANOVA in two factors with replication results, it was observed that SPAD measurement increases significantly with shade ($p < 0.05$), but there was no effect by community (Fig. 12a). Stem length as measure from the base of the stem to highest actively growing leaf showed significant treatment as well as community interaction ($p < 0.05$) (Fig. 12b).



12a. SPAD measurement.



12b. Stem height measured to the apical meristem.

Figures 12a, 12b: Plant growth and physiology. Error bars in 12a and 12b are standard errors from 6 plants per condition. Star indicates significance between two sets ($p < 0.05$) as calculated by students' *t*-test.

CHAPTER 4

DISCUSSION

Gaining a detailed mechanistic understanding of plant microbial community function in natural ecosystem is challenging as there is dynamic interplay of physical, chemical, and biological environment operating at multiple spatial and temporal scales. Numerous species of microbes colonize the plant as members of complex communities occupying different spaces (rhizosphere, endosphere, and phyllosphere) during different season and climatic conditions. Understanding the principles underlying such plant-microbe interaction is problematic as the compositionality and the associated complexity presents a challenge to any experimental analysis.

One possible way to gain insight into the workings of root microbial communities is to establish simplified multispecies communities that can reproducibly colonize the plant. The results presented in the current study provide a robust and taxonomically representative 10-member community of the *Populus* root microbiota, which reproducibly assembles and persists on the roots of axenic *Populus* roots. The data resulting from Experiment II (The temporal dynamics and assembly of constructed community derived from *Populus deltoides* isolates on *Populus* roots determined over the course of 21 days) suggests that the community is reproducible across replicates and hosts at 21 days. Studies beyond the 21-day point would be required to gain more understanding of the dynamics of the constructed microbial community in order to obtain a more comprehensive analysis of microbiomes over time.

In this experiment bacterial strains from *Paraburkholderia* sp BT03 and *Pantoea* sp YR343 were observed in abundance when inoculated as mix. *Pantoea* sp YR343 dominates the community irrespective of length of time, and other inoculated members become part of the community as the days progress. This finding suggests that *Pantoea* and *Paraburkholderia* strains might be occupying different niches and have exclusive purposes for interacting with the host that are free or not dependent on other strains in the inoculated community. Previously, it has been shown that two *Populus*-associated bacterial isolates from *Psuedomonas* and *Paraburkholderia* colonize independently and induce favorable response to the plant-microbiome system in *Populus*⁴³. That observation is corroborated when *Paraburkholderia* sp BT03 and *Pantoea* sp. YR343 are inoculated individually on *Populus*; the comparable strain-specific abundances observed in mono inoculation in this study imply that the two bacterial strains used here might be inhabiting non-competitive niches in the host environment as well. It may also be surmised that, while there is considerable structural and chemical variability within roots that could provide distinctive functional roles for these strains, it is likely that lack of complexity in community composition allows both strains to coexist in the root environment.

Moreover, *Paraburkholderia* species have been shown to produce stress hormones and indole-3-acetic acid (IAA), resulting in the growth promotion of shoots and roots⁸⁸. Plant-associated *Paraburkholderia* strains encode multiple strategies for plant interaction and have also been shown to sense the plant in the environment and respond to stress⁸⁹. *Pantoea* sp. YR343 possesses a number of characteristics that may promote its ability to survive in the root environment and associate with plant hosts,

including both swimming and swarming motility, the ability to solubilize phosphate, and the production of IAA⁸⁸. The individual metabolic ability of each bacterial strain may enable colonization and growth on unique host metabolites.

Experiment III (Determining the potential host specificity of two *Populus* host species towards the constructed community) was a study of two host species that showed bacterial communities did not differ significantly in the composition of inoculated community. Taken together, the results of this experiment suggest that the presence of *Populus* trees did not have a dominant effect over the other factors in determining overall microbial community patterns in the constructed microbiome; that is, genus-specificity in the community composition is still observable. Additional studies that incorporate diverse *Populus* genotypes and co-occurring tree species would be required to fully understand and identify the effects of each factor. It is important to note that the community structure observed is not caused by the plant alone but may also be attributed to the microbial interactions involving cooperation and competition.

In Experiment IV (Determining the colonization of individual member of the constructed community on *Populus* roots) the emergence of *Caulobacter* sp APO7 and *Bacillus* sp. BC15 during mono inoculation suggests that their growth is inhibited when they are present in the mix, or it may be that there is some resource competition with the other members of the inoculated community. Investigating similar effects using microbes that may compete for niche space would determine how specific microbes colonize and compete for the resources within the host and how the plant maintains the microbes. In the assembled 10-member community, the absence of many other microbes may lead to

different interaction networks among the selected members, which probably cause different relative abundance. It would be useful to observe how the strains localize spatially using fluorescent tags so that further knowledge can be gained regarding bacterial-bacterial interactions. However, it is important to acknowledge the primer and DNA extraction procedure bias that is involved in all the measurements as the assumption in this result was that the primer was behaving in a similar manner for all the strains and cells of all the strains equal efficiency, which is unlikely.

Experiment V (The effect of light treatment on constructed communities) studied the end point response of constructed community to the plant subjected to stress in the form of shade. Light limitation treatment is more specifically a host-limited effect that causes differential allocation of carbon. Moreover, it might lead to decreased soil temperature owing to lack of direct light. This experiment found that light limitation did not have any effect on the community composition as measured by the relative abundance of the inoculated strains. However, in respect to plant growth and physiological measurement, although there was no change in the community abundance profile in presence of shade, the plant showed a response by increasing its stem length in reduced light conditions. One explanation for this phenotype is that this is due to shade avoidance syndrome⁹⁰. A plant adapts itself to limiting light and utilizes its resource for its own growth. Increase in foliar chlorophyll concentration in response to shading have been reported in many plants, including *Rhododendron* and *Euonymus*⁹¹⁻⁹². Further work is needed to clarify the relationship between gene expression and metabolite production in order to understand the microbiome interaction with host plant.

This study showed the response of plant-microbiome system to diverse factors such as host and environmental conditions. Nonetheless, the study acknowledges that it is impossible for the 10-member model community to possess all the bacterial interactions and functions of *Populus* root microbiota. Constructed communities do not represent the full breadth and all-inclusive replicate of natural plant microbiome. They have lower complexity compared to those in natural populations so they might miss important community members. Moreover, they do not exemplify true environmental heterogeneity possessing many fluctuating and capricious factors acting concurrently.

However, low complexity and tractable constructed community systems are essential for experimentation to discover causality by targeted manipulation of the system. Constructed community systems permit the recapitulation of microbiome mediated phenotypes that can be verified with alternative communities. Furthermore, they can be used to search for structure and function links in environmental samples in order to validate wide-ranging principles beyond an explicit experimental set-up. New studies and the information could generate a holistic picture of plant-microbiome interaction and fill the existing knowledge gaps.

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