FUNCTIONAL RHIZOSPHERE MICROBIOMES AND EFFECTS ON PLANT-HOST GROWTH, DEVELOPMENT, AND ABIOTIC STRESS TOLERANCE

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FUNCTIONAL RHIZOSPHERE MICROBIOMES AND EFFECTS ON PLANT-HOST GROWTH, DEVELOPMENT, AND ABIOTIC STRESS TOLERANCE

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The rhizosphere microbiome is the community of microorganisms on and surrounding plant roots. This community is important for both above and below ground ecosystem functioning as well as plant growth and development. The depth and complexity of microbe-microbe and plant-microbe interactions within the rhizosphere remain largely uncharacterized. In this dissertation, I explore the rhizosphere system from three directions. First, I propose multiple levels of selection upon extracellular enzyme production and soil organic matter depolymerization as a conceptual framework for explaining the evolution of cooperative rhizospheres. Second, I demonstrate the ability to apply ecosystem-level selection to rhizosphere microcosms to assemble functional microbiomes capable of altering plant flowering phenology and biomass partitioning. I also test the ability of the assembled flowering microbiomes, and sub-communities cultivated from them, to reproduce their function in novel and familiar plant hosts. Flowering microbiomes were able to reproduce their function in several novel *Arabidopsis thaliana* genotypes and *Brassica rapa*, a family-level relative. Cultivated sub-communities displayed variability in their effects on host plant growth and development depending on the composition of the cultivation media. Two of the four cultivation media reproduced the flowering effects of the early-flowering whole microbiome from which they were cultivated. These two sub-communities also increased plant biomass in contrast to the decrease in plant biomass associated with the whole microbiome. Third, I investigate the rhizosphere microbiome of 116

closely-related tall fescue varieties under drought stress to assess the role of the rhizosphere microbiome in genotype-specific variations in abiotic stress tolerance. Differences in drought tolerance were primarily associated with shifts in microbial extracellular enzyme production and fungal endophyte infection rates over differences in bacterial community composition. This work adds to the growing understanding of the complex network of interactions within the rhizosphere and presents ecosystem selection and cultivation as a means of enhancing and characterizing microbiome-mediated effects on plant growth and development. Furthermore, the parallel investigation of rhizosphere microbiome function between plant genotypes and the response of the microbiome to selective pressure begins to uncover the potential of microbial components in traditional plant breeding programs.

BIOGRAPHICAL SKETCH

Kevin Panke-Buisse was born January 13, 1988 and raised in Wisconsin. The natural world has captivated him for as long as he can remember and a passion for agriculture was formed and developed on and around his grandparents' farm. An undergraduate degree at Carthage College provided the opportunity for these interests to intersect with research experience. Kevin participated in several research projects during his time at Carthage and graduated with All-College Honors.

Kevin began a PhD at Cornell University in August of 2010 and served as a teaching assistant within the Department of Horticulture. In 2012, Kevin was awarded a National Science Foundation Integrative Graduate Education and Research Training Traineeship in Cross-scale Biogeochemistry and Climate Science.

For my wife and partner, without whom I would be lost.

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The birth of my son stands out to me as the single most defining moment in my life. His presence provides a stark and piercing window of insight into my character; as a father, partner, human being. His perception of me is a reflection of my purest potential. He provides an aspiration toward which to strive, a compass with which to navigate, and a profound warmth for which there are no words. Through his guidance, I recognize our children to be a biological singularity; simultaneously capable of making us better than we we've ever been and succeeding where we fail.

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PREFACE

Chapter 1 presents extracellular enzyme production in the rhizosphere and soil organic matter depolymerization as one example of plant-microbiome interaction and uses multiple levels of selection as a conceptual framework for understanding the evolution of a cooperative rhizosphere. This chapter will be incorporated into a perspective article for a yet-to-be determined journal.

Chapters 2 and 3 focus on the investigation of functional microbiomes assembled through ecosystem-level selection on the rhizosphere microbiome. Chapter 2 is published in the *International Society of Microbial Ecology Journal* and details the assembly of early- and late-flowering microbiomes and their ability to reproduce flowering effects in novel plant hosts. Chapter 3 investigates cultivation of sub-communities from an assembled early-flowering microbiome and compares the effects of cultivated sub-communities on plant growth in comparison to the whole microbiome. This chapter presents cultivation as a means of pursuing mechanism-level understanding of functional plant microbiomes and altering microbiome function. It is in preparation for submission to *Applied and Environmental Microbiology*.

Chapter 4 explores the composition and function of the rhizosphere microbiome among 116 tall fescue varieties to assess its role in drought tolerance variation between plant genotypes. It is currently in preparation for submission to *New Phytologist*.

CHAPTER 1

EXTRACELLULAR ENZYME DYNAMICS STRUCTURE MORE COMPLEX LEVELS OF PLANT-MICROBE INTERACTIONS

Abstract

The common perception of competition between soil microorganisms and plants for limiting resources is based upon microbial immobilization of limiting nutrients. Recent research on nitrogen mineralization in terrestrial ecosystems is shifting the concept of microbial immobilization away from competition toward cooperation, in scenarios where plants capture the limiting nutrients upon microbial cell turnover. In nitrogen-limited soils, plants play an important role in priming the mineralization of soil organic matter. Microbial growth in bulk soil is often limited by a lack of available carbon. Plant exudation of low molecular weight carbon into the rhizosphere stimulates microbial growth, allowing for greater production of extracellular enzymes. Extracellular enzymes play a critical role in freeing nitrogen and phosphorus bound within complex organic matter. The enzymes are released from microbial cells into the surrounding soil, where they catalyze the breakdown of soil organic matter. We propose that the dynamics of extracellular enzyme production structures more complex levels of plant-microbial interactions that coordinate group-level adaptation to nutrient-limiting soils. We use multi-level selection theory to describe how plants and soil microbial communities co-evolve strategies to favor interactions in rhizospheres as a mechanism for enhanced nutrient capture. Between-group fitness in microbial groups can be favored leading to greater group-level production of extracellular enzymes that enhance the release of limiting nutrients bound within soil organic matter. The limiting nutrient is initially immobilized by the microorganisms and then released for plant and microbial uptake upon cell turnover, akin to slow-release fertilization. Therefore, extracellular enzyme production, as one example of plant-microbe interaction, can serve as a strategy for plants to overcome nitrogen-limiting conditions.

Introduction

Under nutrient limitation, plants and soil microorganisms are perceived to compete for the uptake of nutrients in soil. Once captured by the plant or microorganism, the limiting nutrients become immobilized from the bioavailable pool until they are released through microbial cell turnover, plant tissue senescence, and plant residue degradation. Plants and microorganisms have evolved a cooperative strategy to overcome competition for limiting nutrients through symbiosis. The legume-rhizobia and plant-arbuscular mycorrhizal fungi (AMF) symbioses are typically studied as models for plant-microbial cooperation.[1,2] In both of these examples the partners have been shown to take part in cooperative associations under mutually punitive circumstances where a participant will withhold rewards if it stops receiving "payment" from its partner.[1–3] A less-studied cooperative strategy that does not involve symbiosis is based on group selection involving multiple players that increase their fitness at the community level.

The rhizosphere presents a unique habitat for the coevolution of cooperation between plants and groups of soil microorganisms. Both plant and microbial communities benefit from the release of bound limiting nutrients. Indeed, interactions of microorganisms with their plant host have been shown to be critical to plant health and fitness.[4–6] So much so, that the term "holobiont" has been employed to describe the single adaptive unit formed by a plant and its microbiome.[7,8] In this sense, we suggest that there are three major groups within the rhizosphere: the plant; individual microbial groups; and the microbial supergroup (or the microbiome). These groups can be conceptualized in an economic sense, with each possessing a good for export, and needing to import another: 1) Plants obtain carbon through photosynthesis, but are limited in their ability to catalyze the release of limiting nutrients from SOM; 2) Individual microbial groups lack the full suite of catalytic enzymes to fully mineralize the complex and varied SOM forms; 3) Production of extracellular enzymes is a metabolically costly process and requires both sufficient numbers of producing organisms and a minimum threshold of available materials (C, N, P, etc.) to be feasible. Cooperation between these groups can provide greater overall fitness. Individual microbial groups can cooperate to produce a more complex suite of

extracellular enzymes. Plant tissue residues and root exudates represent sources of the initial accessible C and N required for extracellular enzyme production. In turn, the microbial community can apply its augmented production capacity to acquire C, N, and P for its own and the plant's use.[9–11]

Extracellular enzymes and activity in terrestrial ecosystems

Extracellular enzymes are the vehicle by which soil microorganisms effect decomposition of SOM. Specifically, extracellular enzymes catalyze the depolymerization of essential nutrients from complex forms (chitin, lignin, cellulose) into assimilable forms (ammonium, amino acids, sugars, and mobile phosphate). Soil extracellular enzymes are produced by archaea, bacteria, and fungi, but broadly-applicable methods for determining the fractions of enzymes produced by each group require further investigation to be viable. Identification of enzyme source may be inferable from enzyme pH optima and/or enzyme glycosilation, however these indicators remain too vague to be definitive.[12,13] Enzymes can be classified by the primary nutrient that their action releases; *e.g.* C, N, or P. These nutrients classes can be further subdivided to segregate enzymes by specific substrate and stage of depolymerization. The major enzymes assayed in soil, the substrates and products, and their mechanisms of action are detailed in table 1. Production of soil extracellular enzymes in the context of soil biodiversity is reviewed in Caldwell (2005).[14] The most commonly characterized enzymes are those that catalyze the final step in SOM depolymerization (release of available monomers/substrate), however, degradation of complex molecules often requires several different enzymes that carry out a concerted, staged breakdown of the recalcitrant substrate.[15] For example, hydrolysis of cellulose, one of the best studied substrates in SOM, requires a suite of enzymes that catalyze successive steps in depolymerization beginning with large insoluble B-glucose chains and ending in monomers or dimers available for uptake. The reason for this focus on the terminal depolymerization reaction is that the kinetics are more straight-forward and the products are the assimilable end-product of interest.[15] The implications of extracellular enzyme production in terrestrial systems for global nutrient cycling,

especially in the context of climate change, make it a heavily-studied field. In addition, the inherent complexity of SOM breakdown also makes the soil environment an ideal location for the study of microbial consortia and cooperation.

Extracellular enzymes have a major influence on terrestrial ecosystem dynamics as the agents of microbial nutrient cycling.[16,17] Plants, bacteria, and fungi all have their own mechanisms for the acquisition of phosphorous and carbon; but plants are heavily limited in their ability to take up nitrogen without it first being depolymerized by soil microorganisms.[15] This imbalance factors heavily into N being the foremost limiter of primary productivity in most ecosystems.[18] While some variation does exist between ecosystems and across latitudes, namely the greater tendency towards P-limitation in tropical soils compared to predominate N-limitation in the higher latitudes[19,20], overall N presents both the greatest limitation to ecosystem productivity and the most tightly shared nutrient requirement between plants and the microbial community.

Several studies indicate that microbial communities exhibit the ability to increase enzymatic release of C, N, and P from complex organic matter under conditions of nutrient limitation[21–23], but that enzyme activity is heavily dependent on initial availability of the complex substrate as well as assimilable C, N, and P in the soil.[10,24] Additionally, availability of C, N, and P has been observed to suppress activity of C-, N-, and P-acquiring enzymes[10,22,25,26], leading to the assumption that extracellular enzyme activity is directly related to soil nutrient availability. N fertilization experiments exhibit some variation in their effect on microbial biomass, microbial community composition, extracellular enzyme activity, and decomposition rates, but in general N fertilization decreases N-accessing enzyme activity and can produce either a positive or negative effect on C-accessing enzymes corresponding to the biochemistry of the dominant vegetation.[9,27–30] Specifically, N fertilization tends to increase cellulose activity, but decrease lignolytic enzyme activity[31] and the magnitude and presence of these differences appears to vary strongly between litter and soil.[32] Notable exception can be made for tropical systems which exhibit greater P-dependent

controls of decomposition and patterns of extracellular enzyme production in line with their greater tendency toward P-limitation.[10,33,34]

The traditional view of N acquisition from soil was that it was a primarily microbial process and that the N available to plants was exclusively that NH4 which was not utilized by the microbial community. Recently, the understanding of soil N dynamics has shifted toward an exoenzymatic model that focuses on depolymerization of N from complex substrates, which in turn are freely available to either plants or microorganisms.[35] This shift is thoroughly reviewed and discussed by Schimel and Bennet.[35] The result of this new understanding has been a view of the soil environment as a competitive one; pitting plants against the microbial community for hard-earned assimilable N. This view fails to take into account the potential fitness advantages to mutualistic associations between plants and soil microorganisms.

Rhizosphere priming and nitrogen mineralization

Plant roots significantly alter the soil by the exudation of countless compounds including primary macronutrients like C and N as well as secondary metabolites.[36,37] These exuded compounds span a diverse range of classifications from simple sugars and amino acids, to flavones, quorum sensing mimics, and anti-microbials.[36,38]

The release of labile nutrients into the rhizosphere has been observed to result in a “priming” effect on soil microorganisms. The presence and extent of this priming effect depends heavily on the plant, soil environment, biotic, and abiotic factors.[39,40] While this priming effect is widely observed, the overall influence on N mineralization is inconclusive.[41,42] It must be noted, however, that this priming effect is likely highly specific to growth conditions and the most strongly limiting nutrient in the system. In addition, the majority of studies observe only nitrate and ammonium levels as an indication of N depolymerization. This approach ignores the uptake of organic N by plants, which, as discussed earlier, is an important and often overlooked factor in a complete understanding of below ground N-cycling.[35] Some of the variability in priming effects on SOM decomposition and N mineralization may be explained by these dynamics.[43]

The effect of priming on extracellular enzyme activity has been presented as a method for the disentangling of real and apparent rhizosphere priming effects.[44] Observations of extracellular enzyme activity in response to root exudation has uncovered seasonal variations favoring SOM decomposition in the summer, litter decomposition in the fall, and changes in nitrification rates driven by changes in microbial community functional composition.[45,46] A temperate forest experiment observed increases in N-accessing extracellular enzyme production that differed by tree species and that the form of released N varied by microbial community composition.[47] Summarily, the effects of rhizosphere priming on extracellular enzyme production appear to depend on the stoichiometry of plant root exudates relative to microbial biomass as well as the amount of carbon exuded; i.e. exuded carbon under severe N-limitation cannot result in increases in extracellular enzyme production due to stoichiometric constraints[48] and sufficient levels of carbon exudation must be maintained for an extended period of time to truly stimulate increased extracellular enzyme production and SOM degradation as short pulses favor bursts of microbial turnover rather than stimulated enzyme activity and decomposition.[44] In addition, work comparing the levels of bacterial signaling between bulk and rhizosphere soil have observed a link between increased rhizosphere extracellular enzyme production and increased quorum sensing within the rhizosphere.[49]

Cooperative Rhizosphere

The production of extracellular enzymes provides a major mechanism by which microorganisms gain access to limiting nutrients bound in complex organic matter. Under C-, N-, or P- limiting conditions, groups of microorganisms capable of producing extracellular enzymes are able to capture C, N, and P that would otherwise be inaccessible for biological uptake.[16,26] Microorganisms and plant roots located within hotspots of high enzymatic production benefit from greater availability of C, N, and P. However, there is a fitness cost associated with the production of extracellular enzymes. Producing extracellular enzymes requires both energy and raw materials, primarily in the form of C and N, which would otherwise go into growth and

reproduction. The total cost of extracellular enzyme production alone can incorporate 1-5% of assimilated C and N.[50] Due to the incurred fitness cost, to be evolutionarily viable, extracellular enzyme production should only be conducted when the fitness benefits of capturing C, N, and P bound in organic matter exceed those costs. This principle of required net gain forms the foundation of microbially-mediated decomposition and microbial foraging models.[9,26,51]

Given these considerations, the production of extracellular enzymes and their economic value to the many organisms inhabiting the rhizosphere represents a unique situation in which selective pressures encourage cooperation on multiple levels.[52–54] Owing to the increasing returns of cooperative enzyme production, within-group cooperation and communication is selected-for to allow efficient, economically viable production of enzymes.[55–57] Soil microorganisms capable of producing extracellular enzymes to liberate resources from SOM are selected for greater between-microbe-group cooperation to allow full utilization of the diverse profile of microbial enzymes and potential substrates present within the whole community.[56,58] Plants share the N-, and P-limitation common to the soil microbial community, but do not share the C limitations of their microbial neighbors. This represents an additional level of group dynamic within the soil. Through the exudation of photosynthate and other compounds, plants are able to exert a selective pressure on the rhizosphere community as a whole to promote greater resource mineralization and sharing[59,60], and the rhizosphere community in turn may exert a selective pressure toward plant exudation patterns and phenotypes that best benefit them.[61] In addition, there is the dynamic exchange between individual microbial groups with the plant and among one another. All of these interactions, however, assume a high level of coordination between non-related groups.

Group selection and assortative interactions provide a lens through which to view and conceptualize this cooperative plant-microbe holobiont. [62] In this case, we will define group selection as the application of selective pressures on multiple levels; to both individuals within groups as well as between groups. (Fig. 1) The concept of

assortative interactions assumes that individuals have a degree of choice in with whom they associate. Together with group-level selection of microorganisms, this explains a probable mechanism for the coevolution of highly coordinated/cooperative rhizospheres that enhance nutrient capture.

Coordination of cooperative groups

Given the requirement of net gain for extracellular enzyme production, it follows that evolutionary time would select for microorganisms that minimize the costs, and maximize the benefits, of extracellular enzyme production.[9] Minimizing the cost:benefit ratio can be approached in a variety of ways as seen in the literature and can be separated into three main strategic classes.

1) Regulation of enzyme production to ensure maximum return can be achieved through an inducible pathway that either responds to presence of substrate or suppresses production in the presence of end-product. Most probably, a combination of these strategies is used; e.g. certain bacteria produce enzymes constitutively at a low level, which allows cells to sense the efficiency of their efforts. In the presence of substrate, the cell will sense received product and increase enzyme production accordingly until the resource demand has been met at which point enzyme production can fall back to the constitutive baseline.[22,24]

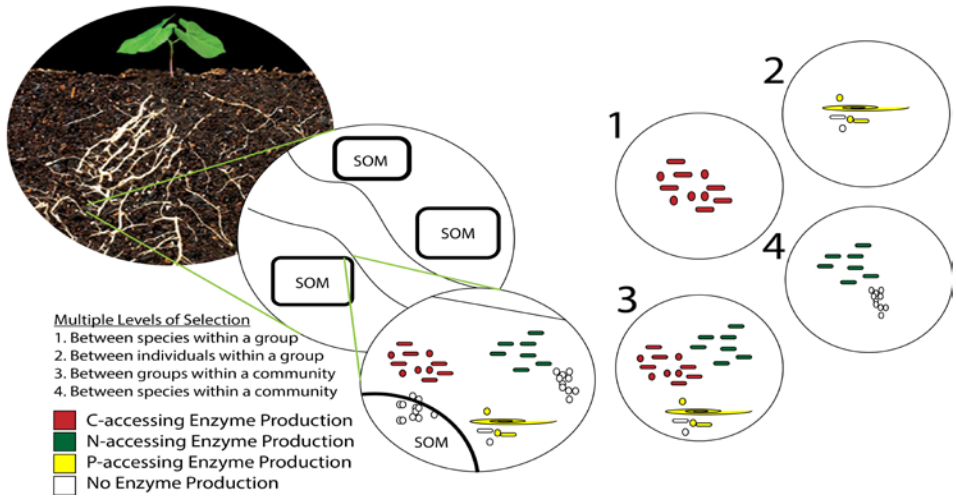

Figure 1. Multiple Levels of Selection Within the Rhizosphere

Conceptual illustration of the rhizosphere and levels of selection occurring within and between microbial groups in the presence of soil organic matter (SOM). The third circle indicates selection between the plant and the microbial community. The circles marked 1 through 4 depict different levels of selection occurring within the microbial community. Clusters of bacteria are meant to represent close associations of microorganisms, or a group, while shapes represent different species. **Circle 1** refers to selection between species within a group. In this case, the group consists of two different species, but all members of the group produce Carbon-accessing extracellular enzymes, **Circle 2** exhibits selection occurring between individuals within a group. Specifically, individuals, regardless of species, do or do not produce phosphorus-accessing enzymes. **Circle 3** illustrates selection between extracellular enzyme producing groups. **Circle 4** shows selection between species (phylogenetic groups) within the community. In this example, two groups of homogenous species composition. Each of these selective pressures can be present concurrently in the microbial community. In addition, selection can occur between the plant and microbial individuals, microbial groups, and the microbial community.

2) Investment protection can minimize the cost:benefit ratio and includes antibiotic production and alteration of the diffusive qualities of produced enzyme to limit benefits lost to cheaters and other competitors.[9,63,64]

3) Extracellular enzyme production is cooperative and includes cooperation, quorum sensing, and biofilm formation.[64,65] It is highly likely that the first two strategic classes co-occur within the third as feedback mechanisms regulating signaling-dependent enzyme production.

Cooperation is a common occurrence in the microbial world, especially among closely related individuals, and signaling between phylogenetically distant species, even trans-kingdom, has been observed on multiple occasions. [2,55] Quorum sensing is a common form of communication that facilitates such coordination within-group and between groups. In order to minimize waste of valuable resources many bacteria exude mall, diffusible molecules constitutively at a low level. As cell density of these quorum sensing bacteria increase, so too does the concentration of the signaling molecule. This system allows bacteria to sense population density and it often regulates genes involved in group behaviors, *e.g*. biofilm formation, virulence, or extracellular enzyme production. The class of quorum sensing molecule best studied is the homoserine lactone family. The length of the carbon chain of these molecules is variable and groups of bacteria have been demonstrated to produce homoserine lactones of a particular length as well as to respond differentially to signaling molecules of different length. This implies an inherent ability to not only use a diffusible proxy for cell density, but also the ability to distinguish between specific signals and respond accordingly. For the illustrated example in Figure 1, this can be conceptualized as the microbial groups possessing the ability to “see” one another’s shapes and colors and interact differently based on that information.

The ideas of bacterial cross-talk and deliberate signaling are not new concepts; however, they provide a mechanism by which cooperation may arise between phylogenetically unrelated groups of microorganisms. Kinship selection is often considered a reliable mechanism for the evolution of altruism in closely related

organisms, but its exclusivity can lead to the assumption that it is the only rigorous theory. Assortative interactions have been shown in simulated models to give rise to high nonrandom variation between non-related groups; conditions that support the evolution of altruistic, and other group-level, traits among individuals. Group selection with assortative interaction models make three key assumptions: 1) the altruistic behavior must be a quantitative, rather than discrete, trait; 2) individuals must have some method of knowing the propensity of other individuals toward the altruistic trait; and 3) individuals must have some level of free choice in association with other individuals. The first is generally considered a safe assumption as few traits in nature are discrete. The second and third are satisfied by the density-dependent, and chain-length specific, signaling, i.e. the altruistic phenotype can be communicated to other altruist individuals via quorum sensing and the specificity of the signal allows individuals a choice in which individuals they associate with.

When the plant is considered, these signaling pathways represent both an avenue for the evolution of cooperative behaviors in the rhizosphere, but also a defense against cheaters. The role of root exudates in communication and effects on soil biotic and abiotic factors is reviewed by Bais et al.[66] If labile carbon and other root exudates can be withheld from undesirable microorganisms or they can be identified by absence/difference of signaling, cheaters may be excluded by the fitness benefits inherent to or the density of the cooperative rhizosphere microorganisms.

Future directions

Further exploration of the concept of microbial immobilization as a coevolved, multi-faceted mutualism between terrestrial plants and soil microorganisms will require further study across the many disciplines embodied within the complex rhizosphere environment.

Teasing apart the interactions between extracellular enzyme-mediated decomposition of SOM and release of SOM-derived N and soil inorganic N has proven difficult in the past. However, with the rapidly increasing understanding of the belowground N-cycle as influenced by microbial community composition and

function, studies focusing on the relative contributions of inorganic N and SOM-derived N to plant and microbial biomass have the potential to elucidate key factors to a more complete understanding of plant-soil-microbial interactions and feedbacks. Advancements in molecular techniques including metagenomics, transcriptomics, biosensors, and DNA/RNA stable isotope probing will allow for more thorough investigation of these questions.[67] Whereas once, questions regarding soil microorganisms were limited to just the small (<1%) that could be isolated and grown in pure culture, these advancements represent culture-independent methods.

Despite the great deal of interest and research into microbial signaling there remains a great deal about microbe-microbe and plant-microbe communication that is unknown. Greater understanding of the methods by which microorganisms communicate and the effects of those communications is integral to pursuing research questions of climate change, agriculture, and ecology. The study of microbial signaling is a highly active area of research and recent work regarding the cooperative association of plants and associated rhizobacteria shows great potential.[2,68] Furthermore, work is being done to link microbial signaling to extracellular enzyme production and depolymerization of SOM N through the use of quorum quenching, or inhibition of quorum sensing.[49,69]

Microbial experimental systems represent an ideal design for the study of microbial community cooperation[70] A long term evolution study has observed the evolution of novel substrate utilization in an experimental population of *E. coli.* [71] This same conceptual model can be combined with or without plants to exert selective pressure toward greater microbe-microbe or plant-microbe cooperation. This technique has already been applied to some degree with drought tolerance, biomass, and flowering phenology as targets of microbial modulation. [72–74] These experimental systems may also allow for a top-down approach to isolation of individual causative or contributive strains by providing a selective filter for the enrichment of such organisms to the point that advanced culture techniques may be applied. [74,75]

Conclusion

The current explanation of interactions between soil microorganisms is primarily one of competition and microbial immobilization. However, recent findings lend support to the idea of cooperative rhizospheres in which plants capture the limiting nutrients upon microbial cell turnover. Plant root exudate patterns can influence the community composition as well as the extracellular enzyme activity of the rhizosphere. Extracellular enzymes may then fulfill their function in depolymerizing nitrogen and phosphorus from complex substrates. This scenario places a unique set of selective pressures on the plant and the microbial community toward increased production and efficiency of extracellular enzymes in the rhizosphere that facilitate the release of labile N and P. While, microbial immobilization of these released nutrients is a problem in agricultural systems in which the short-term immobilization can have negative impacts on crop establishment and overall yield, in the context of natural ecosystems, the time scale of this immobilization may actually represent an adaptive strategy for nutrient storage, similar to a slow-release fertilizer, brought about by the selective pressure toward cooperative fitness in the rhizosphere over evolutionary time. This is only one example of interactions within the rhizosphere and the effect that they can have on plant growth. Other interactions discussed in this dissertation include alteration of complex plant phenology such as flowering time and associations with genotype-specific abiotic stress tolerance.

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CHAPTER 2

SELECTION ON MICROBIOMES REVEALS REPRODUCIBLE IMPACTS ON PLANT FUNCTION

Abstract

Soil microorganisms found in the root zone impact plant growth and development, but the potential to harness these benefits is hampered by the sheer abundance and diversity of the players influencing desirable plant traits. Here, we report a high level of reproducibility of soil microbiomes in altering plant flowering time and soil functions when partnered within and between plant hosts. We used a multi-generation experimental system using *Arabidopsis thaliana* Col to select for soil microbiomes inducing earlier or later flowering times of their hosts. We then inoculated the selected microbiomes from the tenth generation of plantings into the soils of three additional *A. thaliana* genotypes (Ler, Be, RLD) and a related crucifer (*Brassica rapa*). With the exception of Ler, all other plant hosts showed a shift in flowering time corresponding with the inoculation of early- or late-flowering microbiomes. Analysis of the soil microbial community using 16S rRNA gene sequencing showed distinct microbiota profiles assembling by flowering time treatment. Plant hosts grown with the late-flowering-associated microbiomes showed consequent increases in inflorescence biomass for three *A. thaliana* genotypes and an increase in total biomass for *B. rapa*. The increase in biomass was correlated with two- to five-fold enhancement of microbial extracellular enzyme activities associated with nitrogen mineralization in soils. The reproducibility of the flowering phenotype across plant hosts suggests that microbiomes can be selected to modify plant traits and coordinate changes in soil resource pools.

Introduction

Recent studies have highlighted the ability of plant-associated microbiomes to influence plant traits including disease resistance, growth and abiotic stress tolerance.[1–5] When a fast-growing plant is studied in conjunction with its microbiome across multiple generations, new forms of interactions can be observed between plants

and microorganisms shaping plant development. Similar experimental designs using a multi-generational approach have been used to document rapid evolution in plant–insect interactions.[6,7] In the rhizosphere specifically, two recent publications have focused on soil microbiomes to address drought tolerance and disease resistance.[2,8] An earlier study by Swenson et al. (2000) indicates that the microbially mediated mechanisms of plant growth can be passed on through multiple generations of experimental evolution to modulate plant biomass levels. Here, we merge these approaches to examine how selection at the community level (soil microorganisms) for the plant host trait (flowering time) has consistent and reproducible effects on plant function across multiple hosts.

The ability to assemble trait-associated microbiomes into new plant hosts is a critical step in developing an effective microbiome component to plant production systems, particularly for high-value crops that rely on transplantation of young plants into field or greenhouse settings. The ability to progressively enrich for microbiota associated with a specific plant trait facilitates the use of more complex communities instead of a single microbial strain. Related research on disease-suppressive soils illustrates how the concept of multiple plantings of a plant species can enrich for beneficial microbiota that enhance disease resistance in successive generations of plant hosts.[9,10] Transfer of the microbiota into fumigated or sterilized soil containing the young developing plant could help establish persistent plant-associated microbiomes after transplantation to field settings.

In this study, we used a multi-generation approach to generate enriched microbiomes that induce flowering time as the targeted plant trait. Applying community selection on microbiomes through observable changes on a plant trait can demonstrate the potential for complex communities of microorganisms to shape rapid change in plant population traits. We examined the ability of soil microbiomes selected over 10 plantings for progressively earlier or later flowering in *Arabidopsis thaliana* genotype Col to induce the same early- (EF) and late-flowering (LF) times in four novel plant hosts. The soils received low fertilizer inputs to maintain nutrient limitation throughout the study and the soils were autoclaved to facilitate

establishment of the inoculating microbiome into new soils. The central focus of this study features microbiomes from the tenth generation of plantings inoculated into the soils of novel plant hosts that included *Brassica rapa* (BR) and three *A. thaliana* genotypes: Rld, Landsberg erecta (Ler) and Bensheim (Be). We hypothesized that the community selection of microbiomes across 10 generations of earlier or later flowering times in *A. thaliana* Col would result in EF- vs LF plastic responses across all *A. thaliana* hosts and the related *B. rapa* upon inoculation into these novel host soils, and that these microbiomes would differ in phylogenetic composition by flowering time responses.

Materials and Methods

Growth chamber conditions

All plants were grown at 22 °C on a 16/8 h day/night cycle. Relative humidity was set to 75%, and light level was set at 250 μE. (Percival-Cornell University Weill Hall Life Sciences Growth Chamber Facility, Ithaca, NY, USA)

Multi-generation selection of microbiome inoculants

Inoculants for EF- and LF-associated microbiomes were generated through an iterative selection process adapted from Swenson *et al.* (2000).[1] Approximately 100 *A. thaliana seeds* were placed in each of 14 replicate microcosms (7.6 cm diameter × 8 cm height pots) containing 1:1 mixture of field soil: potting mix soil (LM-3 General Purpose Mix; Lambert Peat Moss Inc., Rivière-Ouelle, QC, Canada). The field soil was obtained from a collection of sites across Ithaca, NY, USA (42.456583, −76.368822; 42.452265, −76.369477; and 42.414913, −76.442272) representative of agricultural, forest and grassland ecosystems. The intention was to include a diversity of soil microorganisms for the initial generation. The potting mix was autoclaved for each generation, and became the growing media for the experimental selection. The EF- and LF-associated treatments were established with 14 replicate units each per planting and a control group included 7 units paired with each flowering treatment (14 control units). In each generation, four microcosms were selected based on the highest degree of the plant trait desired. This corresponded with progressively later flowering

or earlier flowering as determined by uniform flower bolting in 90% of the individuals in a unit. Controls were paired with each flowering time microbiome treatment to examine plant traits and soil extracellular enzyme activity results relative to plant phenology. The controls consisted of the plants and steam-sterilized soils, but the units were not inoculated with EF- or LF-associated microbiomes.

Biomass and soils were harvested immediately following flowering of all pots within a group. Loose soil was separated from roots of the four earliest vs four latest flowering replicate units of each treatment group, pooled and mixed with sterile water to form the EF and LF inoculants. Soil slurry inoculants were prepared with 180 ml of sterile deionized water and 30 g of fresh rhizosphere soil, and then shaken vigorously for 60 s upon preparation and periodically during inoculation to make a soil suspension. Each unit for the subsequent generation received 12 ml of the corresponding treatment inoculant. The control group did not receive inoculants of the microbiomes. All seeds across the multi-generation planting were derived from a static seed pool of a highly inbred line, *A. thaliana* Col (Lehle Seed Co., Round Rock, TX, USA). Seeds were derived from this common seed pool to maintain consistent allelic frequencies across all generations and to ensure that any changes in plant traits are the result of microbiome selection. For example, the same pool of seeds was used across generations 1 through 10 and in the EF, LF and control treatments. All microcosms were watered through capillary action using individual reservoirs for each unit. A low level of available nutrients in the potting medium, as well as in the watering regime ensured that the plants were under nutrient limitation, providing a strong filter to impose microbiome effects on soil nutrient mineralization. Fertilizer requirements for *A. thaliana* are high (200 ppm nitrogen (N) every other day) to achieve optimal growing conditions, but we used a fraction of the amount comprising applications of 10 ppm N for generations 1 through 5 for each watering event and three applications of 10 ppm N per generation for generations 6 through 10. As the genetic pool of the plants was held constant, the only adaptive traits to evolve over the iterative generations were derived from the soil inoculation (soil microbial community). This selection process continued for 10 successive generations (plantings) to develop

distinct, trait-associated soil microbiomes associated with EF/LF time.

Transfer of microbiomes to novel plant hosts

Approximately 100 seeds of *A. thaliana* Col, Ler, RLD and Be (Lehle Seed Co.) and six seeds of rapid cycling *B. rapa* (Carolina Biological Supply Co., Burlington, NC, USA) were placed in separate units with 14 replicates measuring 7.6 cm diameter × 8 cm height. Each unit contained the same potting mix used in the multi-generation microbiome selection. The soil mix was autoclaved prior to adding seeds. The EF- and LF microbiomes were inoculated separately into their corresponding replicated units. Each flowering time treatment was paired with a control with seven replicate units. Microorganisms were excluded in the control group inoculants. Each plant host was arranged in the growth chamber in a randomized block design. All units of a plant host received the same amount of fertilizer consisting of a 10% solution (10ppm N, 10.5% nitrate/89.5% urea) of 20-10-20 Jack's Professional General Purpose Fertilizer (J.R. Peters, Inc., Allentown, PA, USA). Plant hosts Be, Col and Ler received three equal doses of fertilizer during growth for a total of 0.9 mg added N, whereas RLD received two doses for a total of 0.65 mg added N, and *B. rapa* received no fertilizer. The difference in fertilizing regimes was due to the rapid flowering, and completion of life cycle, in the EF group for RLD and *B. rapa* in advance of the fertilization schedule and the need to keep nutrient addition constant between treatments.

Plant biomass

Plant aboveground biomass was harvested after flower bolting had begun in 90%of the individuals of each replicate microcosm. Biomass was harvested in two separate portions, reproductive structure and leaf tissue, for the *A. thaliana* genotypes, and whole for *B. rapa*. Harvested tissue was dried at 50 °C until constant weight.

Soil collection and storage

To maximize rhizosphere soil yield, loose soil was removed and soil adhering to the roots was collected. The soil was homogenized and a portion was immediately

frozen at −80 °C for DNA analysis. The other half was analyzed for extracellular enzyme potential activity.

Soil extracellular enzyme activity

We sought to assess microbiome influence on soil processes by measuring the potential activities of soil extracellular enzymes involved in N mineralization. The inclusion of potential extracellular enzyme activity measurements in this study is based on observations of soil nutrient variables established over generations of selection for changes in plant biomass.[1] The enzymes include N-acetyl glucosaminidase, leucine aminopeptidase and phenol oxidase. They function in depolymerizing organic matter and facilitate microbial access to N sequestered within the complex structures.[11] N-acetyl glucosaminidase and leucine aminopeptidase were measured by fluorometric quantification and phenol oxidase was quantified by absorption. We used 4-methylumbelliferone- and 7-amino-4-methylcoumarin-labeled substrates (200 μm), and L-3,4-dihydroxyphenylalanine (25 mm) substrate to provide quantifiable fluorescence and color for quantification of oxidation.[12,13] Soil slurries were prepared from 5 g fresh soil in 150 ml sodium bicarbonate buffer (50 mm, pH 7) and homogenized with an immersion blender for 1 min. Hydrolytic enzyme assays were conducted in black 96-well microplates and oxidative assays were carried out in transparent-bottom 96-well microplates. Standard curves were made for each soil sample (soil slurry+4-methylumbelliferone or 7-amino-4-methylcoumarin standard of 0, 2.5, 5, 10, 25, 50 μm). A 200 μl volume of soil slurry and 50 μl of 4-methylumbelliferone or 7-amino-4-methylcoumarin standards were added into wells of standard plate, and 200 μl of soil slurry and 50 μl of the labeled substrate into wells of substrate plate. Plates were incubated in the dark at 25 °C for 3 h and fluorescence was measured immediately after removal from the incubator with a BioTek Synergy HT microplate reader (BioTek Industries, Inc., Winooski, VT, USA) (ex: 365 nm, em: 450 nm). The oxidative enzyme plate contained a buffer blank (250 μl buffer), a L-3,4-dihydroxyphenylalanine blank (200 ul buffer+50 μl L-3,4-dihydroxyphenylalanine), sample blank (200 μl slurry+50 μl buffer) and the sample wells (200 μl slurry+50 μl L-3,4-dihydroxyphenylalanine). Oxidative plates were incubated in the dark at 25 °C for

3 h and absorbance was measured at 460 nm with the BioTek microplate reader. We calculated activity based on equations from previous work.[12,14]

Microbiome 16S rRNA gene sequencing

Soil DNA was extracted from frozen samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the recommended protocol for highly organic soil. Approximately 0.1 g of soil from each sample was used for isolation of soil DNA. We normalized isolated samples to a concentration of 10 ng ul^{-1} by dilution with PCR-grade water. Quantification was performed with the standard dsDNA quantification protocol for Picogreen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Samples with concentrations below 10 ng ul^{-1} were extracted again at lower elution volume and pooled until a concentration above 10 ng ul^{-1} was reached for normalization. All pipetting for DNA extraction and normalization was conducted with an Eppendorf epMotion 5075 pipetting robot (Eppendorf AG, Hamburg, Germany).

We amplified 16S rRNA gene sequences in duplicate from the extracted DNA. PCR primers used are described in Caporaso *et al.* (2012) that target the bacterial/archaeal 16S rRNA gene variable region 4 (515 F/806 R) for downstream paired-end Illumina (Illumina, Inc., San Diego, CA, USA) barcoded sequencing.[15] We pooled duplicate amplified samples and purified them with the desalting protocol of the Qiagen QiaQuick spin filter purification kit (QIAGEN Inc., Valencia, CA, USA). An epMotion 5075 was used to combine equal concentrations of all barcoded samples and then to dilute the pooled, barcoded amplicons for submission to the Cornell Life Sciences Sequencing Core with the custom sequencing primers as detailed in Caporaso *et al.* (2012) for multiplexed paired-end sequencing on the Illumina MiSeq platform.

Statistics

We used the R statistical package (Rproject.org) and JMP (SAS Institute, Inc., Cary, NC, USA) for all statistical modeling. All manipulations and calculations on 16S rRNA gene sequence data were conducted in the R statistical package. We

modeled biomass, flowering, tissue nutrient and enzyme activity data by standard least squares linear regression with control group values for each response variable included as a covariate to control for the effect of being grown at separate times. The analysis of covariance evaluates each dependent variable across our treatment groups while controlling for covariates. Treatment means adjusted to account for covariates are what are presented in figures to compare differences between the divergent treatment groups. Statistical significances of these comparisons are from the application of a *post hoc* Fisher's test of each plant host, and dependent variable, individually.

Multivariate statistics included multiple linear regression, correlation and covariance matrices to understand the data structure and interactions, and were conducted not only on the biomass, enzyme potential activity and flowering data but also on the relative abundance data of the major phyla/classes. We determined the significance of differences in abundance data by ANOVA (False Discovery Rate-corrected) and significant differences between community composition across groups (LF, EF and Control) were assessed by a nonparametric statistical method, adonis, which identifies relevant centroids, calculates squared deviations and determines significance by F-tests on sequential sums of squares from permutations of data (Quantitative Insights into Microbial Ecology (QIIME) Documentation).

Sequence analysis

Paired-end sequences were truncated at the first low-quality base and quality filtered to remove those with an average quality score below 25, fewer than 200 nt, greater than 700 nt, ambiguous bases, primer mismatches, erroneous barcodes and homopolymer runs exceeding six bases. Paired-end reads were joined and then demultiplexed within the QIIME software package (Qiime.org).[16] We analyzed 16S rRNA gene sequences in the QIIME software tool with the default parameters for each step. *De novo* operational taxonomic unit (OTU) picking was performed with the uclust option in QIIME.[17] Representative OTU sequences were aligned using the PyNAST algorithm with a minimum percent identity of 80%.[18] Assignment of taxonomy to representative OTUs was carried out with the Ribosomal Database Project (RDP) classifier at the default 97%sequence identity and 80% confidence level

with the bundled RDP-assigned taxonomies.[19] Sequences matching plant chloroplast or mitochondrial 16S rRNA were filtered from the dataset.

The heatmap was created from the log abundance of all genera and classified by the Prediction Analysis for Microarrays for the R package, which uses the least shrunken centroid method.[20] The ternary plot was created with ggplot2 in R.

We determined the optimal sampling depth through examination of exploratory rarefaction curves of observed species plotted against sampling depth and the dataset was rarefied to 12 000 sequences per sample. Samples with fewer reads were removed. Alpha diversity metrics (chao1, PD, observed species) were computed within QIIME. Distance matrices were generated with the unweighted and weighted UniFrac methods to compare the relative abundance and presence/absence patterns between treatment groups. Our beta diversity measures (between-sample diversity) were computed with QIIME and jackknifed by repeatedly sampling at 3000 sequences per sample. Beta diversity was then plotted by principal coordinates analysis with confidence ellipses generated from the jackknifing procedure.

Results

Soil microbiome composition

Soil microbiotas obtained from the root zone of *B. rapa* and four *A. thaliana* genotypes grouped together primarily by flowering time treatment and controls. Figure 1 shows a heatmap of log absolute abundance for all taxa. The samples grouped specifically by EF, LF and control (C) treatments. The ‘control’ serves as a profile of the surviving and residual microbiota endemic in the soils after steam-sterilization and without inoculation of additional microbiota. Although the heatmap showed strong clustering by treatment, eight samples were misclassified representing an error rate of 0.075.

Distribution of OTUs across samples revealed a core microbiome with 60% of all OTUs shared across flowering time and control treatment groups (Figure 2a). The center of the ternary plot shows the core microbiome (high density of circles) across the EF, LF and control treatments. The OTUs uniquely associated with a specific

treatment (where more than 80% of the total abundance of a particular OTU is uniquely associated with only one group) corresponded to the points within the corners of the ternary plot. The genera assigned to these OTUs fall into a handful of key families (Figure 2b). The bacteria most strongly associated with the EF treatments include genera within two families with many known plant pathogens

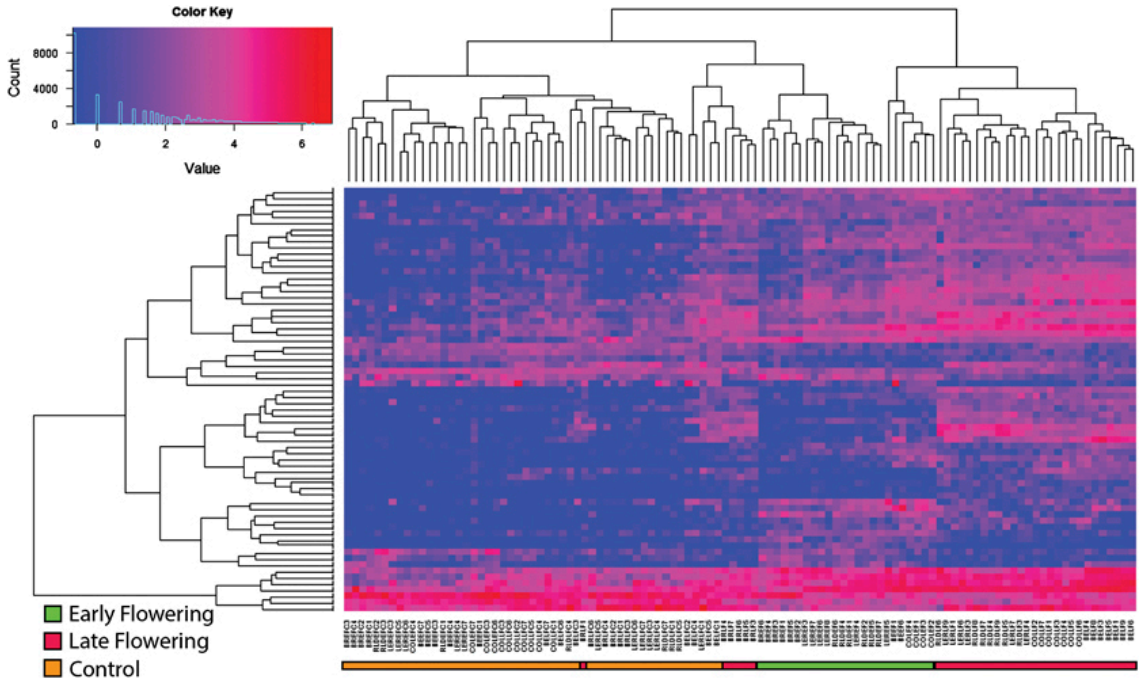
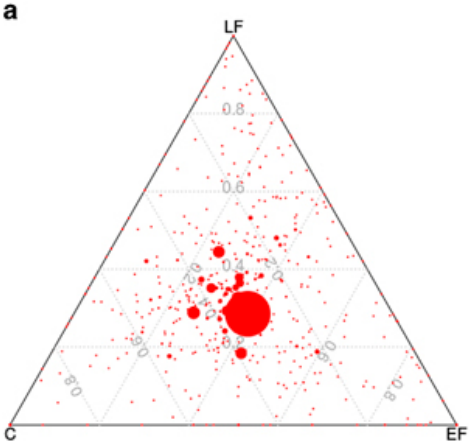


Figure 1. Soil microbiota group together primarily by flowering time treatment and controls. Heatmap of log absolute abundance of all taxa. Classification, dendrograms and order of samples and taxa were determined by the Prediction Analysis for Microarrays in the R statistical package. The color key at the top left includes a frequency histogram of number of OTUs at each expression level. Vertical columns represent samples mapping primarily into 'Control', EF and LF treatment groups. The 'control' serves as a profile of the surviving and residual microbiota endemic in the soils after steam-sterilization and without inoculation of additional microbiota. Although the heatmap showed strong clustering by treatment, eight samples were misclassified representing an error rate of 0.075.

a



b

EF	C	LF
Cellulomonadaceae	Acidobacteriaceae	5B-12
Clostridiaceae	Armatimonadaceae	Alcaligenaceae
Geobacteraceae	Beijerinckiaceae	Caldilineaceae
Hydrogenophilaceae	Chthoniobacteraceae	Conexibacteraceae
Lachnospiraceae	Chthonomonadaceae	Corynebacteriaceae
Methylocystaceae	Erythrobacteraceae	Dolo_23
Methylophilaceae	Flexibacteraceae	Enterobacteriaceae
Moraxellaceae	Gemmatimonadaceae	Iamiaceae
Paenibacillaceae	Ignavibacteriaceae	Leptospiraceae
Pseudomonadaceae	koll13	Sporichthyaceae
R4-41B	Marinilabiaceae	Verrucomicrobiaceae
Rickettsiaceae	Patulibacteraceae	WD294
Saprospiraceae	Procabacteriaceae	
Spirochaetaceae		
Xanthomonadaceae		

Figure 2. Family-level OTUs uniquely associated with EF/LF time groups and controls. (a) Ternary plot of OTUs showing the percent of each OTU's observations present in each group (EF, LF and Control) across different plant hosts. For example, a point's position within the '0.8' triangle at the 'EF' corner of the ternary plot indicates that 80% of all observations of that OTU occur within the EF group. Diameter of plotted points corresponds to relative abundance of the OTU. Compartments of the dotted grid correspond to 20%increments. (b) List of taxonomy at the family level corresponding to OTUs of points falling within the 80% compartment of each group.

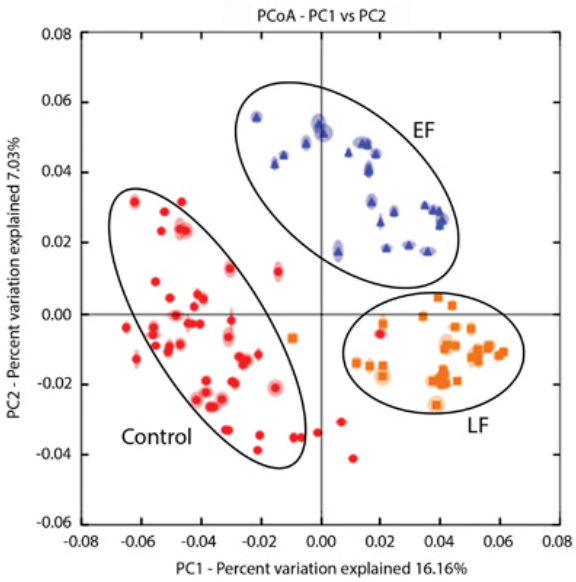


Figure 3. Unweighted UniFrac distances show separation of the EF/LF-associated microbiome treatments and controls by microbial taxa. Principle coordinates analysis of unweighted UniFrac distances generated from 16S rRNA gene sequence data obtained from the rhizosphere soils of the plant hosts. Unweighted UniFrac distances are insensitive to relative abundance of observed OTUs and instead reveal patterns and differences in the presence/absence of taxa. Samples were rarefied to an even sampling depth of 12 000 seqs per sample. The orange points refer to LF microbiomes, the blue points are the EF microbiomes and the red points are the control microbiomes. Percentages on each axis represent the percent variation explained by the PCs.

(Xanthomonadaceae and Pseudomonadaceae) and genera within three families with members associated with nutrient mineralization and substrate depolymerization (Moraxellaceae, Cellulomonadaceae and Saprospiraceae).[21–24] In contrast, the LF treatments were associated with families that include plant-growth-promoting bacteria (Iamiaceae, Alcaligenaceae and Corynebacteriaceae) and a family of bacteria (Verrucomicrobiaceae) that are ubiquitous in soil but are poorly represented through culturing methods.[25–28]

Principal coordinates analysis of the unweighted UniFrac distances showed separation of the trait-associated microbiome treatments (EF vs LF) and the control by microbial taxa (Figure 3). In contrast, the weighted UniFrac analysis indicated no separation of flowering time and control microbiomes in this study. Although the majority of soil studies place emphasis on the relative abundance of taxa, to which weighted UniFrac is sensitive, the multiple generations of selection in this study may have led to the enrichment of trait-associated rare taxa—meaning abundant taxa alone may not drive the observed differences in flowering time. Furthermore, patterns of presence/absence can be obscured by the high relative abundances of core microbiome taxa, making unweighted UniFrac (insensitive to relative abundance) better for elucidating these patterns.[29]

Effect of selected microbiomes on plant host traits

When the EF- and LF-associated microbiomes were inoculated into soils containing novel plant hosts, we found consistent responses in differences between flowering times. All *A. thaliana* hosts grown with LF-associated microbiomes flowered 15–17% later than plants containing the EF-associated microbiomes. The related crucifer, *B. rapa*, flowered 56% later in the LF treatments than in EF treatments. The significant delays in flowering were associated with increases in inflorescence biomass of three of the four *A. thaliana* genotypes Col, RLD and Be. Similarly, *B. rapa*, also showed delayed reproduction and an increase in total aboveground biomass in the LF-associated microbiome treatment (Figures 4a and b).

Potential microbiome-mediated shifts in soil environment

Soil microbial communities have a strong role in biogeochemical processes that determine soil environmental parameters such as pH, mineralization and nutrient availability.[30,31] We observed no significant changes in soil pH between treatments and plant hosts, which indicates that pH is not responsible for the observed differences in plant growth and phenology. Soil inorganic NH_4^+ and NO_3^- concentrations did not differ across treatments, but any differences generated from mineralization could be explained by rapid immobilization in soil microorganisms and plants. Although aboveground plant tissue N did not differ across flowering treatment groups, it is plausible that root tissue N differed (but root tissue N was not measured in this study). The potential soil extracellular enzyme activities associated with N mineralization increased two- to fivefold in the LF microbiome treatment over the EF microbiome treatment (Figure 4c). Enhanced extracellular enzyme activity can indicate increased microbial coordination in the depolymerization of complex substrates and release of bioavailable N or phosphorus.[32,33]

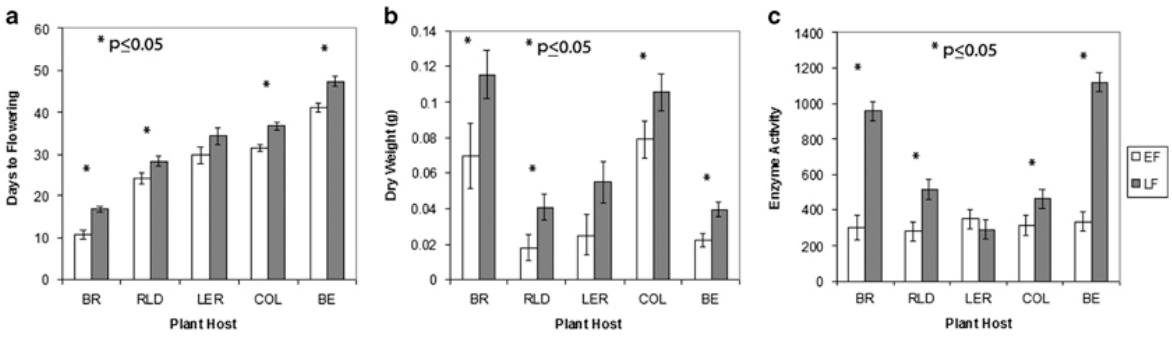


Figure 4. Flowering time, reproductive biomass and potential extracellular enzyme activity at harvest show consistent changes across plant hosts. (a) Days to flowering of each plant host after inoculation with EF and LF microbiomes. (b) Average reproductive biomass (grams dry weight) for the A. thaliana genotypes and total biomass for B. rapa. (c) Potential extracellular enzyme activity (umol $hour^{-1}$ gram dry $weight^{-1}$) in soils across plant hosts. Enzyme activity associated with N mineralization is represented by the sum of leucine aminopeptidase, N-acetyl glucosaminidase and phenol oxidase (Sinsabaugh, 2010). Enzyme activity is measured in nmol per gram soil per hour. Values reported are from a standard least squares regression model including control values as a covariate (analysis of covariance). Plant host abbreviations correspond to B. rapa (BR) and the four A. thaliana genotypes Rld (RLD), Ler (LER), Col-0 (COL) and Be (BE). Asterisks denote statistical significance at $P<0.05$. Error bars represent s.e.m.

Discussion

Although the mechanisms underlying the apparent microbiome-driven shifts in flowering time are unknown in this study, we speculate that microbial modification of the soil altered a suite of environmental cues controlling flowering time. Regulation of flowering time is primarily driven by abiotic factors, such as vernalization and photoperiod, but it is well known that stress and nutrient availability also influence flowering time.[34,35] The increase in *A. thaliana* reproductive biomass and *B. rapa* total biomass associated with the LF microbiomes points to several possibilities that include changes in soil resource pools altering flowering time, delayed reproduction altering soil resource pools and accumulated pathogens that contribute to early stress flowering. In this study, the delay in flowering corresponded to a 50–100% increase in host reproductive or total biomass. Minor increases in bioavailable N or other limiting nutrients could result in the biomass gains observed in the plant hosts particularly because the plants in this experiment were grown under nutrient limitation.

The production of extracellular enzymes provides a major mechanism by which microorganisms gain access to limiting nutrients bound in soil organic matter. Under N- or phosphorus-limiting conditions, groups of microorganisms capable of producing extracellular enzymes are able to capture N or phosphorus that would otherwise be inaccessible for biological uptake.[30,36,37] These groups of microorganisms may include both bacteria and fungi, although fungi were not specifically examined in this study because of the lack of mycorrhizal association in *A. thaliana a*nd less robust community profiling methods. Plant rhizodeposition and root exudates represent a potential catalyst needed to prime the breakdown of complex polymers that release mineralized N and phosphorus.[38] Given this beneficial association, the production of extracellular enzymes and their value to the many organisms inhabiting the rhizosphere represent a unique situation in which selective pressures may encourage higher level coordination between plants and their.[39–41]

It is conceivable that the multi-generational approach to microbiome assembly we used may have led to the development of microbiomes in the LF treatment that enhance N mineralization *via* extracellular enzyme production. The resulting increase

in mineralized N could modulate nutrient stress responses thereby favoring delays in bolting. Reproductive delay in *A. thaliana* grown in low phosphorus soils has been shown to increase biomass by 30% presumably by allowing greater time for soil phosphorus mineralization and root exploration.[42] Similarly, in the study by Swenson *et al.* (2000), continuous selection for high vs low biomass *A. thaliana* plants showed changes in soil chemistry. By generations 13 and 14, phosphorus was one of the major factors explaining the separation of soil nutrients by low vs high host biomass selection lines.

The ability to reproduce microbiome function in novel plant hosts suggests that microbiome composition is also reproducible. However, inoculation of a plant's root-associated microbiome into the soils of novel plant hosts does not necessarily lead to a reassembly of microbial communities representative of the inoculant. For example, legumes inoculated with a mixture of rhizobial strains showed that nodule formation with the effective strain was not achieved uniformly across legume genotypes.[43] In this study, we showed that the plant trait-associated microbiomes developed over multiple generations were able to assemble into distinct community profiles by flowering time treatment across novel plant hosts. Although the soils were steam-sterilized to reduce viable microorganisms, a small fraction of the community is still able to persist as found in other studies.[8] In spite of the persistence, the inoculated microbiomes were able to populate the soils of novel hosts and induce plastic responses in flowering phenology and soil function. While bacterial sequencing was emphasized in this study as in other plant microbiome-focused papers[44–46], fungi could have had a significant role in modulating flowering time, altering extracellular enzyme activities and enhancing reproductive biomass. Root-colonizing endophytic fungi and root-associated fungi are able to modulate stress and enhance plant growth in *Arabidopsis* and other hosts.[47,48] Although the multi-generation approach to enriching microbiomes is likely to favor bacterial populations, it is conceivable that certain fungi are enriched across plantings assuming that fungal hyphae were able to persist through the inoculation procedure we used and establish in host tissues or rhizospheres within the

short lifecycle of the rapidly cycling Brassicas.

Irreproducibility of microbiome function in Landsberg Erecta

The *A. thaliana* genotype Ler showed microbiome profiles consistent with the other plant hosts, but was unable to show the same significant shifts in flowering time, biomass and soil extracellular enzyme activities. Genotypic variability within a species can influence the composition of plant-associated microorganisms. For *A. thaliana*, a study conducted on eight genotypes in two different soil types showed that genotype explained a small but significant fraction of variation in the composition of the endophytic microbiome.[44] Similarly, a study conducted on maize genotypes showed that a similar fraction of variation in rhizosphere microbial diversity was explained by plant host genetics.[46] In our study, the lack of a significant response found in Ler to the inoculated microbiomes could be related to variation in host genetics. In particular, Ler shows unique genetic traits relevant to flowering regulation that could contribute to a reduced ability to delay flowering. For example, LF associated with the FRIGIDA (FRI) gene is partially suppressed in Ler and the suppressor allele found in Ler (FLC-Ler) may constrain the expression of the LF phenotype through inhibiting increases in Flowering Locus C (FLC) expression.[49]

Conclusion

We have shown that experimental selection on soil microbial communities can alter major plant traits, including flowering time. These trait-associated microbiomes can then populate the soils of novel hosts and reproduce their intended functions. The ability of microbiomes to reproduce their effects on soil processes and host plant traits is critical to advancing the use of soil microbiomes in plant production systems. Our findings from the sequencing analysis indicate that rare taxa may have important roles in plant trait development. Accordingly, our results suggest that selection based on diverse microbial communities holds strong potential for using microbiomes to address key agronomic and environmental concerns.

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CHAPTER 3

CULTIVATED SUB-POPULATIONS OF MICROBIOMES RETAIN EARLY FLOWERING PLANT TRAIT

Abstract

The collection of microorganisms found in the root zone of soil, termed the rhizosphere microbiome, has been shown to impact plant growth and development. Here, we tease apart the function of the cultivable portion of the microbiome from the whole microbiome in retaining plant traits modified through artificial selection on flowering time. Specifically, the whole microbiome associated with earlier flowering time of *Arabidopsis thaliana* was cultivated on four types of solid media to create cultivated fractions of the microbiome. These cultivated microbiomes were subsequently preserved in glycerol, frozen, and revived to yield a portion of the cultivable fraction to compare: 1) whole microbiome, 2) cultivable microbiome, and 3) revived cultivable microbiome controls on early flowering time. Plants grown in soils inoculated with bacteria grown on 25% Luria broth, 10% tryptic soy agar, and pseudomonad semi-selective agar retained the early flowering trait. An increase in leaf biomass with two of the cultivated microbiomes contrasted the lower biomass effect of the whole microbiome. Inoculation with the cultivated microbiomes that were cryopreserved in glycerol showed no effect on flowering time or leaf biomass. The results indicate that the cultivable portion of a plant's microbiome retains the early flowering effect in *A. thaliana*, but cryopreservation of the cultivated microbiomes disrupts the microbial controls on flowering time. Furthermore, the contrasting effects on leaf biomass (an indirect response from selection on early flowering time) seen with the whole microbiome versus the cultivable portion suggests versatility in using cultivation methods to modify multiple traits of plants.

Introduction

Several studies have emerged exploring the potential of plant-associated microbiomes to influence a wide range of traits including growth, disease suppression, stress tolerance, and flowering.[1–4] Traditionally, most investigations of host-microbe interactions tend to look for and isolate one or a handful of strains responsible for a change in host phenotype. While isolates are convenient for studying host-microbe interactions, recent work suggests that complex microbial communities, from consortia of just a few to whole microbiomes, may show more robustness in

altering complex host traits.[4–7] Increasingly, more studies are examining the whole-microbiome effects on plant host traits.[7–9] This line of inquiry has led to greater interest in complex host-microbe and microbe-microbe interactions.[5,10,11] Whole-microbiome investigations are not without their constraints, however. The complexity of whole microbiomes makes identification of the actual players driving the host responses difficult. Cultivation of whole-microbiomes without isolation of single strains is presented as a method to reduce the complexity of the microbial communities while retaining the key microbial players and preserving their cooperative functions

The ability to assemble functional microbiomes is an important step to expanding both fundamental and applied research into plant-microbiome interactions. Microbiome assembly is primarily approached from the bottom-up relying on the addition of candidate taxa by either phylogenetic or functional genotypic/phenotypic criteria.[12] Alternatively, we used directed selection in a previous study to assemble plant microbiomes associated with flowering time of multiple plant hosts, and found distinct bacterial community profiles assembling by flowering time using 16S rRNA gene sequencing.[7] This represents a departure from existing methods by applying a top-down approach that replaces strain or species function with whole-microbiome function as a selection criterion. Interestingly, microbiomes also exhibited effects on secondary traits (not directly selected-for), including differences in biomass. The novelty of the study was the ability to generate microbiomes using a cultivation-independent approach and transferring these microbiomes into the soils of new hosts to reproduce the selected plant trait. However, the role and interactions of the key microbial players remained elusive even with robust sequencing data (12,000 reads per sample). This was due in part to the fact that shifts in flowering time corresponded more to patterns of presence/absence of bacterial taxa than patterns of relative abundance. This emphasis occurred because many taxa appeared in all of the samples, indicating a shared, core microbiome that likely had little to no effect on flowering time.

In this study, we examined an early-flowering microbiome enriched over 15 generations of selection, in comparison to subsets of strains cultivated from fresh soil and cryo-preserved cultivates for their ability to reproduce effects on plant traits in *A. thaliana*. Cultivated fractions were derived from soil and water mixtures incubated on four solid media. Revived microbiomes were derived from cultivated microbiomes preserved in glycerol at -80˚C. Flowering was considered the primary trait modulated by the microbiome and cultivated microbiomes, while

other attributes such as rhizosphere pH and plant biomass were considered secondary traits. Comparison of the primary and secondary trait effects of whole microbiomes, cultivated microbiomes, and revived microbiomes can demonstrate the potential of using sub-populations of microbiomes to modify plant traits. We hypothesized that inoculation of the early-flowering cultivable subsets into *A. thaliana* soils would reproduce the flowering response, but that variation in microbiota across the different growing media and cultivation methods would lead to variations in the secondary plant trait (biomass) indirectly selected on through the multi-generation study.

Materials and Methods

Growth chamber conditions

Plants were grown at a constant 22°C under a 16hr/8hr day/night cycle in a growth chamber. (Percival-Cornell University Weill Hall Life Sciences Growth Chamber Facility, Ithaca, NY) Light intensity at plant height was 250 UE m^{-2} $s^{-1.}$ Relative humidity was set to 70% for the duration of the study.

Plant growth conditions

All seeds across all phases of this study came from a static seed pool of a highly inbred line, A. thaliana Col (Lehle Seed Co., Round Rock, TX). Seeds were used from this common seed pool to fix allelic frequencies across all phases of this study and to ensure that any changes in plant traits when compared to controls or other phases of the study are the result of microbiome inoculation. All microcosms were watered from bottom reservoirs.

Assembly of early flowering microbiomes

Inoculants for early-flowering microbiomes were generated through an iterative selection process detailed previously.[7,13] Microcosms (n=14, 7.6 cm diam. pots) of ~100 *A. thaliana* individuals grown in a 1:1 mixture of field soil: potting mix soil (Lambert General Purpose Mix) were prepared. The field soil was obtained from a collection of sites across Ithaca, NY (42.456583, -76.368822; 42.452265, -76.369477; and 42.414913, -76.442272) representing agricultural, forest, and grassland soils. The mixed environment soil was added to provide a

diversity of soil microorganisms for the initial generation. The potting mix was autoclaved prior to each generation, and after the first generation, it became the sole growing medium for the remainder of the study. In each generation, a subset of soil from the four pots displaying the earliest flowering was set aside as the inoculant for the next generation. Biomass and soils were harvested immediately following flower bolting of >90% of the individuals within all pots. Control pots consisted of the plants and steam-sterilized soils inoculated but the units were not inoculated with early- or late-flowering microbiomes.

In this study, the subsets of soil for inoculation were pooled and "inoculant slurries" were prepared by combining 180 mL of sterile, deionized water and 35g of the harvested rhizosphere soil, then shaken vigorously for 60 seconds upon preparation and periodically during inoculation. The autoclaved soil in each pot of the subsequent generation was inoculated with 12 mL of the slurry. The control group pots were treated with a sterile inoculant. Plants were watered with a 10% solution (10ppm N, 10.5% nitrate/89.5% urea) of 20-10-20 Jack's Professional General Purpose Fertilizer (J.R. Peters, Inc., Allentown, PA, USA). The low level of available nutrients in the potting medium, as well as in the watering regime ensured that the plants were under nutrient limitation, providing a strong filter to impose microbiome effects on soil nutrient mineralization. As the genetic pool of the plants was held constant using a highly in-bred genotype, the only adaptive traits to advance over generations were limited to those derived from the soil inoculation (of trait-associated microbiota). This selection process continued for 15 successive plantings to develop distinct, trait-associated soil microbiomes associated with early flowering time.

Cultivation

Cultivation methods were employed to test the ability of the cultivable fraction to reproduce the function of the early flowering microbiome. Inoculant slurries for cultivation were prepared by combining 30g of trait-associated rhizosphere soil from each of the four pots that displayed earliest flowering and 25 mL of sterile, deionized water in a 50 mL tube, shaking for one hour. Soil was pelleted at 3500 x g for 30 minutes and 750 uL of supernatant was inoculated onto each of five replicate plates and spread using a flame-sterilized glass spreader. The plates were incubated at 25ºC in the dark for seven days. Glycerol stocks (25%) of all plates were made from a swipe and stored at -80ºC for the revival portion of the study.

The four solid media (25% Luria broth (LB), 10% tryptic soy agar (TSA), pseudomonad selective agar (PSA)[14], and rhizosphere medium (RM) were prepared according to the recipes in Table S1. The "rhizosphere medium" was prepared by blending rhizosphere soil with agar (50% soil by volume) and autoclaving. Selective agents (antibiotics and cycloheximide) were filter sterilized and added after autoclaving soils, immediately prior to pouring. Previous work (data not shown) supported the bacterial community as the drivers of flowering time differences. Cycloheximide was added as an antifungal agent to discourage dominance of fungi during cultivation.

Reproduction of function in cultivated fractions of early-flowering microbiomes (from fresh soil)

In order to test the effects of cultivated subpopulations on plant growth, cultivated fractions were inoculated into a plug flat containing Lambert General Purpose Mix. A streak from each plate was taken and suspended in 2 mL of phosphate-buffered saline (PBS). Plugs were inoculated with 60 uL of the mixture. The surface of the plug flats were sprayed with sterile water to saturate the potting soil. After 48hr, five *A. thaliana* Col seeds were sown into each plug. Control plugs were inoculated with either sterile water or PBS to establish a baseline of growth for the native soil microbiome and to account for the addition of PBS. The different treatment and control plugs were situated within the flats at random. Domes were added to create high humidity conditions until germination and establishment, after which they were removed. Flowering times were recorded following the complete flower bolting of a microcosm. Both leaf and reproductive tissue biomass were harvested, and dried at 50˚C until constant weight.

Reproduction of function in revived microbiomes of cryo-preserved cultivates

The frozen glycerol stocks were revived for both liquid and solid cultivation. For the liquid cultivation method, glycerol stocks were inoculated into 1mL of the respective medium in which they were originally cultured, without selective agents (antibiotic and antifungal) or agar. These were then incubated for 4 hours at 25ºC. Starter cultivations of 250uL were then transferred to 5mL liquid cultures containing the selective agents detailed in Table 1. For the plate method, inoculant was retrieved from the glycerol stock and placed into 200 mL of the respective medium, incubated for one hour, and plated onto the respective solid medium,

complete with selective agents. Two replicates were prepared for each glycerol stock sample (solid and liquid), and all cultivations were incubated at 25ºC in the dark.

Cultivated microbiomes were incubated for 5 days and were inoculated randomly into plug flats of sterile potting mix as described above. Growing conditions and sample collection were as described in the previous section. For the plate method, a streak of the plate colonies was suspended in PBS. Then, 60uL of either liquid cultivation or a PBS-slurry of the solid medium cultivation was inoculated into each plug. Duplicates of each replicate were inoculated to mitigate error from edge effects and microclimatic variation. Control plugs were inoculated with either sterile water or PBS and were also randomly placed.

16S rRNA gene sequencing

Soil DNA was extracted from frozen samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the recommended protocol for highly organic soil. Approximately 0.15 g of soil from each sample was used for isolation of soil DNA. Quantification was performed with the standard dsDNA quantification protocol for Picogreen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All pipetting for DNA extraction was conducted with an Eppendorf epMotion 5075 pipetting robot (Eppendorf AG, Hamburg, Germany). We amplified 16 S rRNA gene sequences in duplicate from the extracted DNA. The PCR primers used are described in Caporaso et al. (2012) that target the bacterial/archaeal 16 S rRNA gene variable region 4 (515 F/806 R) for downstream paired-end Illumina (Illumina, Inc., San Diego, CA, USA) barcoded sequencing.[15] Amplicon were quantified with Picogreen and 200ng of each sample were pooled and purified with the desalting protocol of the Qiagen QiaQuick spin filter purification kit (QIAGEN Inc., Valencia, CA, USA). Amplicon pool was submitted to the Cornell Life Sciences Sequencing Core with the custom sequencing primers detailed in Caporaso et al. (2012).

Statistics and sequence analysis

Plant trait data were analyzed by Analysis of Variance (ANOVA) using JMP 10 (SAS Inc.). Significance between groups was determined at an α-level of 0.05. Contrasts between means were found using post-hoc analysis (Fisher LSD and Tukey's HSD).

For 16S rRNA gene sequence analysis, paired-end reads were truncated at the first low-quality base and quality filtered to remove those with an average quality score below 25, fewer than 200 nt, ambiguous bases, primer mismatches, erroneous barcodes, and homopolymer runs exceeding six bases. Paired-end reads were joined and then demultiplexed within the QIIME software package (Qiime.org).[16] Operational taxonomic units (OTUs) were picked *de novo* by clustering similar sequences with uclust.[17] Sequences with sequence identity below 60% and sequences matching plant chloroplast or mitochondrial 16 S rRNA were filtered from the dataset. The smallest number of sequences belonging to any sample was 9799. This value was used to rarify all samples to that number of input sequences for analysis requiring even samples sizes for robust results. Alpha diversity measures (within-sample diversity) were calculated with Strong's dominance.[18] Beta diversity measures (between-sample diversity) were computed with weighted UniFrac and the resulting distance matrix was used to create the principal coordinates plot.[19] The heatmap of key taxa was created from the log abundance of all orders that differ significantly between samples exhibiting early-flowering/biomass shifts and those that did no. These were then classified by the Prediction Analysis for Microarrays for the R package, which uses the least shrunken centroid method.[20] Log2-fold-change and significance of taxa shifts were computed using the DESeq2 method.[21] The inputs used for DESeq2 were only taxa present in >80% of the samples of a given phenological group (*e.g.* early-flowering vs. no flowering effect), in other words, the core microbiomes associated with the plant phenotype effects.

Results

Whole microbiome phase

Inoculation of whole early-flowering microbiomes into soils of *A. thaliana* genotype Col led to decreases in leaf biomass and fewer number of days to flowering, when compared to a sterile inoculant control. Reproductive biomass was unaffected by treatment in all three phases of the experiment. Individual plant leaf biomass in the treatment group was decreased by 61.2% from the control group: 0.0047 $\pm$ 0.0014 and 0.0124 $\pm$ 0.0022 respectively (Figure 1B). Similarly, flowering time decreased in the treatment group by 9.1% compared to the control group: 30.15 $\pm$ 0.38 and 33.17 $\pm$ 0.63 days respectively (Figure 1A).

Cultivable microbiome phase (cultured from soil)

Cultivated microbiomes exerted significant differences on both plant leaf biomass and days to flowering from control microcosms and one another. PBS was used as an isotonic solution to suspend cultivated inoculants prior to inoculation. The PBS-inoculated controls and sterile inoculant controls were compared to determine if the addition of PBS altered plant growth. PBS showed no effect on plant growth (data not shown). Flowering responses in the culturing phase were characterized by significant flowering times: 8.7% and 10.9% earlier than the control for LB and TSA media, respectively, and 4.7% percent later for RM (Figure 1A). Leaf biomass was characterized by significant increases of 49.4% and 38.5% for LB and TSA media, respectively (Figure 1B).

Revived microbiome phase (from cryo-preserved cultivated microbiomes)

Revived microbiome inoculation yielded no significant differences in plant biomass from control microcosms. In addition, no flowering effect was observed for any of the treatment groups, indicating a complete loss of treatment effect. PBS had no effect on plant growth. (Data not shown).

Control comparisons

Phases were analyzed independently of one another due to the difference in microcosm size between the whole microbiome phase and the culturing and revival phases. In order to ensure the robustness of comparing results between phases, we also compared control groups across all phases. There was no significant difference between control groups across phases for either flowering time or leaf biomass.

16S rRNA gene sequencing analysis

Bacterial community patterns show visible shifts in taxa abundance between the whole and cultivated groups (Figure S1). The whole microbiome and LB cultivated groups show significant variation within-group as well. Principle coordinates analysis (PCoA) illustrates the within-group variance in the whole microbiome and LB groups, the uniformity of the TSA, PSA, and RM groups, and the relationship between all of the treatment groups (Fig. 2). The results of the DESeq2 analysis produced a list of only 197 OTUs that differ significantly between samples

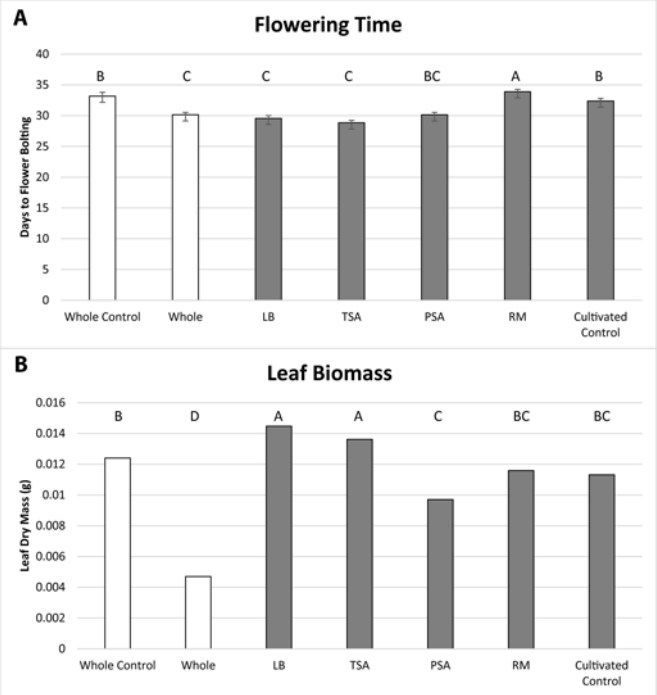

Figure 1. Inoculant Effects on Flowering Time and Leaf Biomass

Comparison of whole microbiome and cultivated microbiomes' effects on (A) flowering time (days to flower bolting of > 90% of individuals) and (B) leaf biomass (dry weight). Each phase, whole microbiome and cultivated microbiome have distinct controls in which plants were inoculated with sterile soil (whole microbiome) or sterile water (cultivated microbiome). Whole microbiome phase bars are unshaded and cultivated microbiome phase bars are shaded gray. Media abbreviations - LB: 25% Luria Broth; TSA: 10% Tryptic Soy; PSA: Psuedomonad Semi-selective; RM: Rhizosphere.

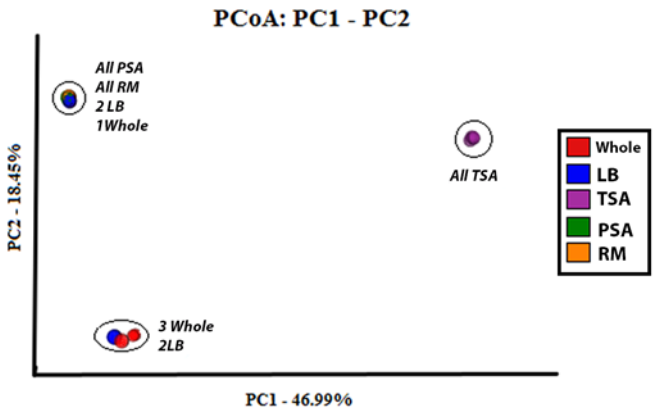

Figure 2. Weighted UniFrac Principal Coordinates Plot

Principal coordinates plot of weighted unifrac distance matrix illustrates the similarities and differences within and between sample groups. Weighted UniFrac distances show separation of the microbiome treatments by microbial community composition. Weighted UniFrac distances are sensitive to relative abundance of observed OTUs and reveal patterns and differences in the abundance of taxa. Samples were rarefied to an even sampling depth of 9799 seqs per sample based on the sample with the smallest number of sequences. Percentages on each axis represent the percent variation explained by each of the PCs. Close proximity of points obscures individual colors. Circles have been added around clusters and sample points within each cluster are listed adjacent to each cluster.

exhibiting an early flowering effect and those that did not (Figure S2). In addition, the shift from low biomass to high biomass was characterized by significant shifts in only 31 OTUs (Figure S3). These 228 “key taxa” were used as the input for the PAMR heatmap to visualize the shifts between groups (Fig. 3).

Discussion

We show that cultivation of mixed strains or taxa from rhizosphere soil presents potential benefits over the use of single isolate methods to modify a plant trait, but approaches to preserve and revive these cultivable microbiomes resulted in a loss of the trait effect. Specifically, the inability to maintain the flowering and biomass effects through cryopreservation and revival of the cultivated microbiome is likely a function of poor survival of taxa associated with these plant traits and selection for taxa that are tolerant of cryopreservation.[22,23] The cultivable microbiome, while less complex than the whole microbiome, appears to retain a sufficient portion of the microbial community responsible for the early-flowering effect. The loss of this effect in the revived cultivable microbiome indicates the need to study the role of complex communities in plant-microbiome interactions. In addition, inoculation of the cultivated sub-populations into the soils containing *A. thaliana* led to unexpected changes in plant flowering and leaf biomass responses.

Previous speculation on the driving processes behind microbiome-mediated shifts in flowering time include alteration of environmental cues tied to flowering (photoperiod and vernalization), pathogen pressures, and nutrient availability stresses.[4,7,24] Results of this study, however, suggest that the factors driving flowering time modulation may not be so straightforward. The whole microbiome treatment was characterized by significant decreases in flowering time and leaf biomass, which is consistent with low-nutrient or non-lethal pathogen accumulation stress responses.[25] However, two of the cultivated microbiomes (grown on LB and TSA) retained roughly equivalent decreases in flowering time, but exhibited ~40-50% increases in leaf biomass in comparison to controls. These observations suggest that the plant-microbe-environment interactions that induce the primary early-flowering response are not necessarily linked to the secondary leaf biomass response. The increase in biomass observed in the cultivated microbiome phase is consistent with previous studies on initiation of flowering in *A. thaliana*.[26,27] This may indicate that cultivation of the early-flowering microbiome can eliminate

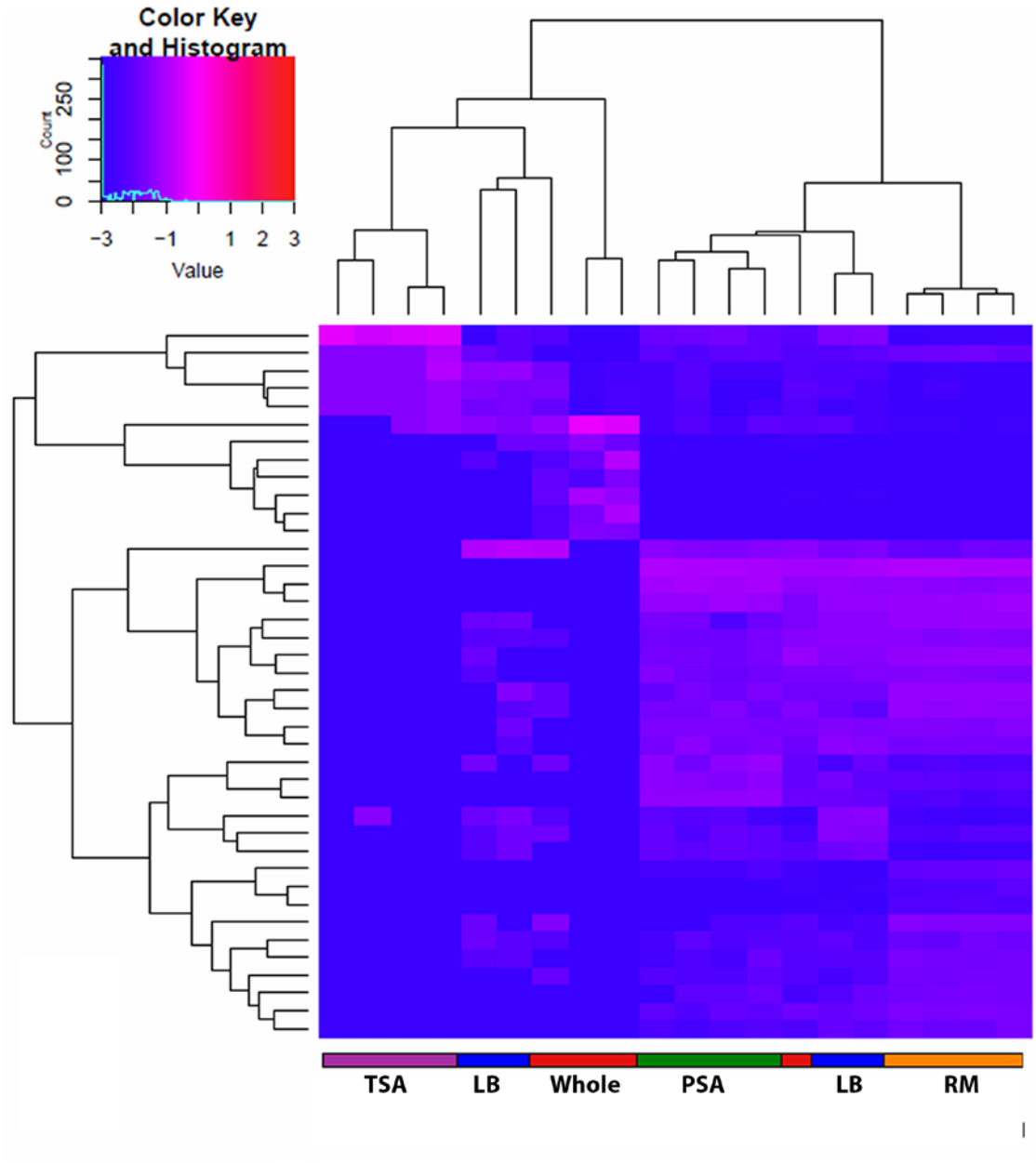

Figure 3. PAMR Heatmap of Key Taxa

Heatmap of log relative abundance of key OTUs associated with observed phenotype effects identified by DESeq2. Columns represent individual samples and cluster primarily by treatment group. The rows represent OTUs at the order level. Dendrograms on each axis illustrate the relationship between the columns/rows. The color key at the top left includes a frequency histogram of number of OTUs at each log expression level. OTUs with zero expression were changed to 0.001 to allow the use of a log transformation. The whole microbiome and LB groups are the only two groups from which samples do not cluster correctly. 2 LB samples and 1 whole microbiome sample do not group with their corresponding treatments.

deleterious members of the microbiome responsible for the decrease in leaf biomass in the whole microbiome. Furthermore, decoupling of the early-flowering and low-biomass traits could potentially suggest a more direct role of the microbiome in flowering time modulation.

The differences in biomass and flowering responses between cultivation media present cultivation as a strategy for eliminating undesirable taxa from microbiomes. Cultivation and inoculation into a sterilized rhizosphere can disrupt existing associations between microorganisms of the whole microbiome and potentially change interactions with the new plant host. Similar disruption of established plant-microbe associations has been observed to change overall community function.[28] In this regard, choice of cultivation medium and plant host appear to play a crucial role in determining microbial succession dynamics within the new host rhizosphere and, in turn, overall function in the plant host. This is supported by the observation of larger, more dominant community members in the whole microbiome treatment and smaller, more numerous members in the cultivated treatments, as determined by alpha diversity indices.[18]

Community analysis revealed that the taxa shifts apparently driving these effects come down to a very small percentage of the overall community. The early-flowering effect is characterized by shifts in only 197 key taxa, including overall decreases in abundance of certain Actinobacteria, Acidobacteria, Bacteroidetes, Proteobacteria, and Verrucomicrobia and increases in Spirochaetes, Firmicutes, and the Archaea Crenarchaeota (Class MBGA) (Fig. S2). The biomass effect was characterized by shifts in just 31 key taxa between the low and high biomass effects (Fig. S3). The high biomass effect was represented by relative increases in select Firmicutes, Bacteroidetes, Spirochaetes, Proteobacteria, and the Archaea Crenarchaeota (Class MBGB) and relative decreases in Actinobacteria. Only 6 of these 228 taxa are associated with both effects. Furthermore, many of these taxa are virtually unstudied and lie outside the traditional plant growth-promoting groups that typically include Psuedomonads, Rhizobia, Azospirillum, Bacillus, Streptomycetes, Azotobacter, and *Agrobacterium*.[29]

Taken together, these results highlight cultivation as a method for simplifying microbiome communities while retaining, enhancing, or modifying microbiome function. A lack of mechanistic understanding currently limits studies of bio-inoculant efficacy for commercial production.[30] The associated reduction in complexity and changes in cultivated microbiome effects between cultivation media supports the utility of using sub-populations of the microbiome in pursuing mechanism-level understanding of plant-microbe interactions. Cultivation may also

contribute to the development of novel technologies and processes for plant production systems. Current methods of commercial bio-inoculation can be readily adapted for use with more complex cultivated microbiomes over single-strain inoculants.[31] The biomass differences observed between plants grown with the whole and cultivated microbiomes illustrate the potential of cultivation for maintaining primary plant traits of a microbiome and modulating secondary traits. For example, a microbiome that accelerates time to flowering and increases plant biomass is well-suited for agriculture and could reduce production time and costs. Finally, based on our microbial community profile data, cultivation and subsequent reintroduction to the rhizosphere appears to allow for the transfer and enrichment of taxa that cannot be isolated in culture.

Conclusion

We have presented the cultivation of functional microbiomes here as a potential tool for further study of the microbe-microbe and plant-microbe interactions involved in the elicitation of desired plant responses offering unique advantages over whole-microbiome and isolation/culture-based approaches. The ability of cultivated microbiomes, derived from the early-flowering microbiome, to both reproduce the primary plant response (flowering time) and modulate the secondary response (leaf biomass) contributes strongly to evidence in support of microbiome-use in plant production systems. Specifically, it presents an experimental platform for refining functional microbiomes to facilitate mechanism-level understanding of their interaction with plants.

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Supplement Table and Figure Legends

Table S1. Recipes for Solid Media

The preparation instructions for the four solid media used in this study. Cycloheximide was added as an antifungal agent to discourage dominance of fungi during cultivation. Previous work (data not shown) supported the bacterial community as the drivers of flowering time differences.

Figure S1. Histogram of Relative Abundances Summarized by Group

Bar chart of relative abundance of phyla summarized within groups. Each color represents a phylum and its size corresponds to the relative abundance of that phylum within the treatment group. Distinct visual patterns are present between treatment groups. Media abbreviations - LB: 25% Luria Broth; TSA: 10% Tryptic Soy; PSA: Psuedomonad Semi-selective; RM: Rhizosphere.

Figure S2. Log2-fold Change in Abundance of Flowering-associated Taxa

Key taxa were identified by analysis with DESeq2 differential abundance analysis. Only taxa present in >80% of the samples that showed an early-flowering effect and those present in >80% that showed no flowering effect were used as inputs for DESeq in order to assess the core microbiome. Relativized log (Log2-fold Change) bars are grouped by phylum to assist in delineations between taxa groups.

Figure S3. Log2-fold Change in Abundance of Biomass-associated Taxa

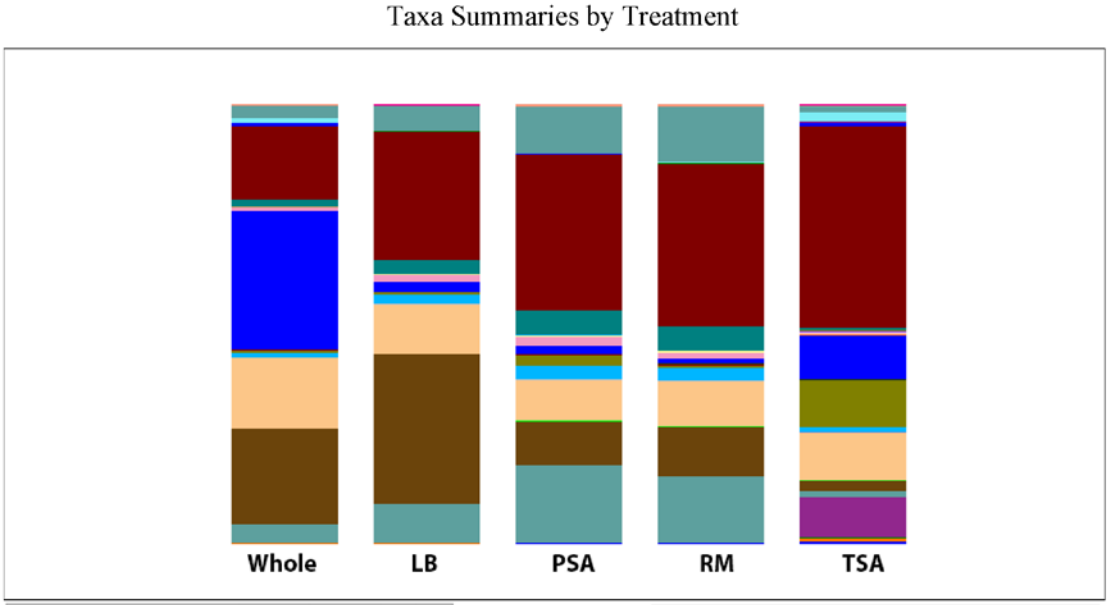
Key taxa were identified by analysis with DESeq2 differential abundance analysis. Only taxa present in >80% of the samples that showed a low biomass effect and those present in >80% that showed a high biomass effect were used as inputs for DESeq in order to assess the core microbiome. Relativized log (Log2-fold Change) bars are grouped by closest shared taxonomic level to assist in delineations between taxa groups. Taxa preceded by: “c__” are classes, “o__” are orders, and “f__” are families.

Table S1. Recipes for Solid Media

The preparation instructions for the four solid media used in this study.

¼ Luria Broth	1/10 Tryptic Soy Agar	Pseudomonad Selective Agar	Rhizosphere Media
2.5g Tryptone 0.8g Yeast Extract 2.5g NaCl 15g Agar 10ug/mL Cycloheximide	1.7g Tryptone 0.3g Soy Flour 0.5g NaCl 0.25g Dipotassium Phosphate 0.25g Glucose 15g Agar 10ug/mL Cycloheximide	20g Peptone 1.4g MgCl 10g KSO4 10mL Glycerol 13g Agar 40ug/mL Ampicillin 12.5 ug/mL Chloramphenicol 10ug/mL Cycloheximide	50g Arabidopsis Rhizosphere Soil (blended for homogeneity) 0.5g Dipotassium Phosphate 1g glucose 15g agar 10ug/mL Cycloheximide

Supplementary Figure 1. Histogram of Relative Abundances Summarized by Group

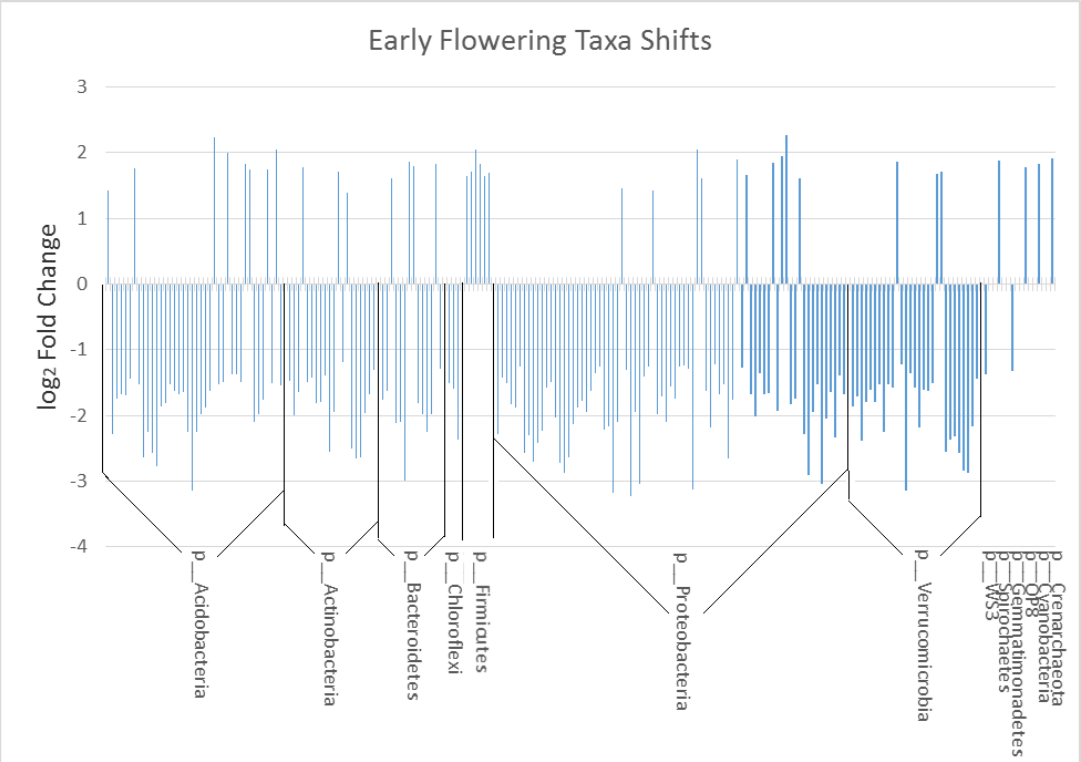


Legend	Taxonomy	Total %	Full %	LB %	PSA %	RM %	TSA %
	k__Archaea;Other	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Archaea;p__Crenarchaeota	0.2%	0.1%	0.1%	0.2%	0.1%	0.4%
	k__Archaea;p__Euryarchaeota	0.3%	0.2%	0.0%	0.0%	0.0%	1.0%
	k__Archaea;p__[Parvarchaeota]	0.0%	0.0%	0.0%	0.0%	0.0%	0.2%
	k__Bacteria;Other	1.9%	0.1%	0.1%	0.1%	0.1%	9.1%
	k__Bacteria;p__	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__AC1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__AD3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Acidobacteria	9.4%	4.1%	8.8%	17.5%	15.1%	1.3%
	k__Bacteria;p__Actinobacteria	15.9%	21.7%	34.1%	10.0%	11.1%	2.3%
	k__Bacteria;p__Aquificae	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Armatimonadetes	0.2%	0.1%	0.1%	0.3%	0.1%	0.1%
	k__Bacteria;p__BHI80-139	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__BRC1	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%
	k__Bacteria;p__Bacteroidetes	11.5%	16.1%	11.2%	9.0%	10.2%	10.8%
	k__Bacteria;p__CD12	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Caldiserica	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Calditrichx	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Chlamydiae	0.1%	0.1%	0.1%	0.2%	0.2%	0.0%
	k__Bacteria;p__Chlorobi	0.1%	0.1%	0.1%	0.1%	0.2%	0.1%
	k__Bacteria;p__Chloroflexi	2.0%	1.0%	1.9%	3.0%	2.8%	1.0%
	k__Bacteria;p__Chrysiogenetes	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Cyanobacteria	2.9%	0.5%	0.5%	2.3%	0.5%	10.7%
	k__Bacteria;p__Deferribacteres	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Dictyoglomi	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__EM3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Elusimicrobia	0.2%	0.1%	0.1%	0.2%	0.3%	0.0%
	k__Bacteria;p__FBP	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%
	k__Bacteria;p__FCPU426	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Fibrobacteres	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%
	k__Bacteria;p__Firmicutes	9.3%	31.4%	2.2%	1.9%	1.1%	9.9%
	k__Bacteria;p__Fusobacteria	0.0%	0.1%	0.0%	0.0%	0.0%	0.2%
	k__Bacteria;p__GN01	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__GN02	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%
	k__Bacteria;p__GN04	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__GOUTA4	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Gemmatimonadetes	1.2%	0.8%	1.7%	1.9%	1.3%	0.2%
	k__Bacteria;p__H-178	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Hyd24-12	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__KSB3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

Legend	Taxonomy	Total %	Full %	LB %	PSA %	RM %	TSA %
	k__Bacteria;p__LD1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Lentisphaerae	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__MVP-21	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__MVS-104	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__NC10	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__NKB19	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Nitrospirae	0.2%	0.1%	0.2%	0.4%	0.4%	0.1%
	k__Bacteria;p__OD1	0.1%	0.0%	0.1%	0.1%	0.0%	0.1%
	k__Bacteria;p__OP1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__OP11	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%
	k__Bacteria;p__OP3	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%
	k__Bacteria;p__OP8	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%
	k__Bacteria;p__OP9	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%
	k__Bacteria;p__PAUC34f	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Planctomycetes	3.3%	1.7%	2.9%	5.6%	5.5%	0.6%
	k__Bacteria;p__Proteobacteria	32.9%	16.7%	29.2%	35.5%	37.0%	45.8%
	k__Bacteria;p__SAR406	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__SBR1093	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__SR1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Spirochaetes	0.3%	0.7%	0.0%	0.0%	0.0%	0.6%
	k__Bacteria;p__Synergistetes	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%
	k__Bacteria;p__TM6	0.2%	0.1%	0.2%	0.1%	0.2%	0.2%
	k__Bacteria;p__TM7	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%
	k__Bacteria;p__TPD-58	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Tenericutes	0.7%	1.0%	0.0%	0.1%	0.1%	2.1%
	k__Bacteria;p__Thermotogae	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%
	k__Bacteria;p__Verrucomicrobia	6.6%	2.9%	5.6%	10.6%	12.5%	1.3%
	k__Bacteria;p__WPS-2	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%
	k__Bacteria;p__WS1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__WS2	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__WS3	0.3%	0.1%	0.2%	0.5%	0.5%	0.0%
	k__Bacteria;p__WS4	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__WS5	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__WS6	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__WWE1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__ZB3	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__[Calditrichx]	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__[Thermi]	0.1%	0.1%	0.2%	0.0%	0.0%	0.3%

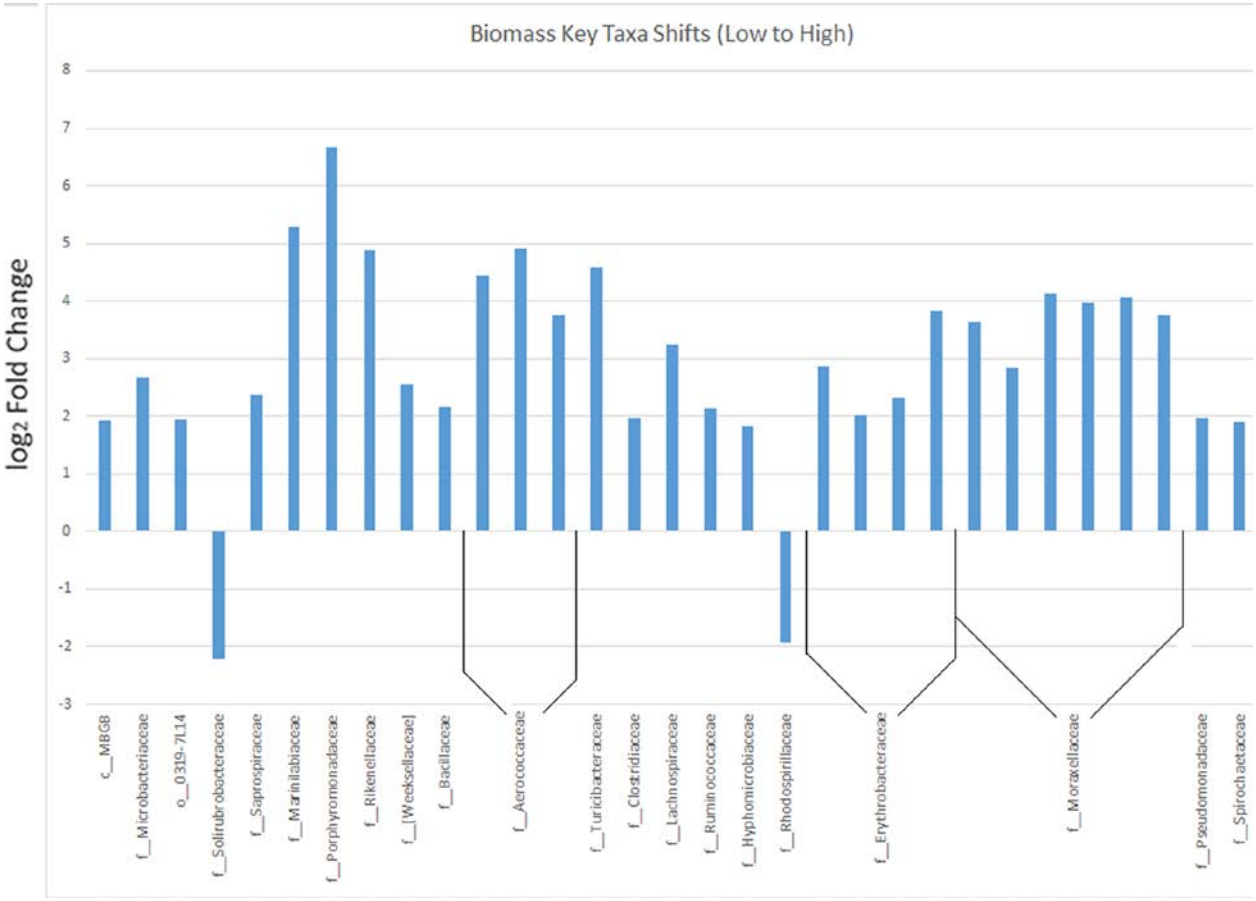
Bar chart of relative abundance of phyla summarized within groups. Each color represents a phylum and its size corresponds to the relative abundance of that phylum within the treatment group. Distinct visual patterns are present between treatment groups. Media abbreviations - LB: 25% Luria Broth; TSA: 10% Tryptic Soy; PSA: Psuedomonad Semi-selective; RM: Rhizosphere.

Supplementary Figure 2. Log2-fold Change in Abundance of Flowering-associated Taxa



Key taxa were identified by analysis with DESeq2 differential abundance analysis. Only taxa present in >80% of the samples that showed an early-flowering effect and those present in >80% that showed no flowering effect were used as inputs for DESeq in order to assess the core microbiome. Relativized log (Log2-fold Change) bars are grouped by phylum to assist in delineations between taxa groups. Significance is determined at alpha-level 0.05 of false discovery rate corrected p-values.

Supplementary Figure 3. Log2-fold Change in Abundance of Biomass-associated Taxa



Key taxa were identified by analysis with DESeq2 differential abundance analysis. Only taxa present in >80% of the samples that showed a low biomass effect and those present in >80% that showed a high biomass effect were used as inputs for DESeq in order to assess the core microbiome. Relativized log (Log2-fold Change) bars are grouped by closest shared taxonomic level to assist in delineations between taxa groups. Taxa preceded by: “c__” are classes, “o__” are orders, and “f__” are families. Significance is determined at alpha-level 0.05 of false discovery rate corrected p-values.

CHAPTER 4

MICROBIOME FUNCTION AND DROUGHT TOLERANCE OF TALL FESCUE GENOTYPES

Abstract

Plant response to abiotic stress can be altered by the soil microbial community, but the role of the soil microbiome in plant genotype-specific tolerance is unclear. Here, we report the role of the soil microbial community on drought tolerance in tall fescue. We imposed drought conditions on 116 tall fescue genotypes and then irrigated plots to stimulate recovery. Soil extracellular enzyme activities and microbial community profiles were assessed at three points: initial, peak drought, and post-recovery. 16 S rRNA sequencing revealed microbial communities that were largely identical regardless of plant genotype or drought tolerance. Microbial community composition was driven primarily by experiment phase (Initial, Drought, Recovery) and shifts in the Recovery phase were characterized by domination of phyla Proteobacteria and Bacteroidetes. Trends in dark septate endophyte infection rates in roots pre-drought revealed that higher infection rates resulted in negative impacts on drought tolerance. Extracellular enzyme activity during drought also differed significantly with drought tolerance. Contrary to expectation, highly drought tolerant genotypes exhibited lower rates of root infection by soil fungal endophytes and higher phosphatase and phenol oxidase extracellular enzyme activities. These observations suggest that despite few clear differences in microbial community composition, function differs significantly between drought tolerance groups. This presents plant genotype-specific alteration of microbiome function as an explanation for microbial community contribution to abiotic stress tolerance.

Introduction

Microbiome influence on plant growth and development is a growing area of active research. Previous articles have demonstrated a role for the microbiome in drought tolerance.[1,2] However, identifying plant genotype-specific shifts in the soil microbial community has proven elusive. Plant genotype effects on microbial community composition are often obscured by the large shifts provoked by environmental conditions.[3–6] Here, we grouped genotypes by their

performance in a functional category (drought tolerance) to investigate the role of the microbial community in population-level variation of that trait.

Tall fescue is a grass species that readily associates with fungal endophytes, including mycorrhizal fungi and dark septate fungal endophytes, and is of high economic and environmental value for its potential to withstand climate change impacts.[7] The interest in tall fescue as a drought tolerant turf and forage species has led to the production of many cultivated varieties (genotypes), and a large body of research that characterizes physiological[8], nutritional[9], and symbiotic[10–12] aspects of drought tolerance. This makes tall fescue an ideal model for investigations of soil microbial shifts corresponding to drought tolerance.

Although foliar endophytes of tall fescue have been well studied, there is considerably little known about root fungal endophytes and the soil bacterial community. In particular, investigations of dark septate fungal endophytes (DSE) in tall fescue are lacking. DSE have been shown to be comparable to mycorrhizal fungi in terms of abundance and function and there is evidence of DSE modification of mycorrhizae growth.[13–16] DSE are presented in this study as an indicator of the endophytic portion of the eukaryotic microbial community. Better understanding of the structure and function of the rhizosphere microbiome (including both fungal endophytes and bacteria), in relation to drought, could help elucidate microbiome mediation of drought tolerance among tall fescue genotypes. Identifying patterns of microbial community structure and function specific to grass genotype tolerance to drought would support the development of microbial community phenotyping as a selection trait in plant breeding programs, as a means to help reinforce the expression of desirable plant traits that include drought tolerance, among others. Microbial components of plant systems provide an additional target for addressing the challenges posed by climate change and food production.[17–20] Tall fescue specifically, as a drought tolerant turf and forage grass, is well-suited to addressing these challenges.

This study follows 116 tall fescue genotypes planted in a sandy loam under simulated drought. Genotypes were categorized as low-, or medium-, or high-tolerance in response to the drought. Plant health, bacterial community composition, and extracellular enzyme activity were assessed at pre-drought, time-zero (Initial), during peak drought (Drought), and following recovery (Recovery), with the goal of identifying key shifts corresponding to drought tolerance. The enzymes chosen play roles in depolymerizing organic matter and facilitating microbial access to

carbon, nitrogen, and phosphorus sequestered within complex organic compounds and represent an estimate of microbial community function.[21] Dark septate endophyte infection rates were measured at time-zero. We hypothesized that drought tolerance would correspond to any combination of three primary microbially mediated factors: 1) microbiome composition, 2) dark septate endophyte infection rate, and 3) extracellular enzyme activity.

Materials and Methods

Design and field conditions

A total of 116 genotypes of tall fescue were established in Arkport sandy loam in 2012 at the Cornell University Bluegrass Lane field research site in Ithaca, NY. Plants were mowed to a height of approximately 7.62cm when height exceeded 4.5in. Nitrogen was applied 2-4 times per year at a rate of $\leq$ 1.22g N/m^2/growing month. To initiate the simulated drought period, the experimental area was saturated with 2.5cm of irrigation per day for three consecutive days to create uniformly wet conditions. A moveable rainout shelter was used to exclude rainwater from plots while still allowing light penetration. This shelter was deployed over plots during rain events and withdrawn under dry conditions to prevent excessively high temperatures beneath the shelter. Drought was imposed on the plots in July 2014 and ended 15 days after the first genotype in September 2014 was less than 20% green by area. Following the drought, a recovery period was initiated during which the plots received 5cm of irrigation to stimulate drought recovery and 2.5cm of irrigation per week thereafter until the study's conclusion.

Tall fescue genotypes were assigned to low-, medium-, or high-tolerance groups based on DGCI values. Volumetric water content at peak drought was used to identify plots that received water during the drought through edge effects or rainout shelter leaks (>9%). After elimination by water content, there were 112 genotypes remaining with the high-tolerance group containing 20 genotypes, the low-tolerance group containing 26 genotypes, and the medium tolerance group containing the remaining 66.

Field measurements and sampling

Digital photographs were taken with a lightbox and camera at the start of drought, peak drought, and weekly during recovery. These photographs were analyzed for percent dark green cover (DGCI) with Sigmascan Pro (Systat Software, San Jose, California, United States) [22,23].

Volumetric water content of the soil was taken with a Field Scout TDR 300 moisture probe (Spectrum Technologies, Inc., Aurora, IL, United States). Soil samples were obtained for downstream analyses at the start of the experiment, at peak drought, and at the end of the recovery period with a 0.5 inch soil sampling probe. Two ten centimeter cores were obtained from each plot, above ground biomass was removed, and the cores were homogenized and stored at -20C.

Fungal endophyte quantification

The full root systems from each soil core were carefully washed under running tap water to remove soil particles and then placed into the 1-mm pore Biopsy cassettes (Tissue Tek®, Electron Microscopy Sciences). Root samples were stained the using an established ink-vinegar method.[24] Briefly, roots were cleared in 20% (w/v) KOH at room temperature for 40 minutes. They were then washed with tap water and transferred to distilled white vinegar (5% acetic acid) for at least 3 hours to ensure they were adequately acidified for staining. Acidified roots were transferred to 5% ink-vinegar solution (Parker Quink Permanent black-blue ink: distilled white vinegar in the ratio of 95:5) to stain for 30 minutes and then a quick dip (~ 1 second) into 0.5% KOH to remove excess staining. 15 to 20 stained roots per sample were mounted on slide with PVLG (a mixture of 100 ml lactic acid, 100 ml water, 10 ml glycerol and 16g polyvinyl alcohol powder). Each slide was scanned methodically at 200·magnification (increased to 400 for precise examination) for at least of 150 intersection per sample. The following fungal structures indicative of DSE infection were recorded: (1) fungal hyphae with frequent septa and melanized walls and (2) microsclerotia consisting of clusters of melanized circular or elliptical cells. DSE infection rate was quantified as the percentage of observations with DSE structures [25].

Soil extracellular enzyme activity

Potential soil extracellular enzyme activity was used as a measurement for microbial function in substrate depolymerization. The enzymes assayed include N-acetyl glucosaminidase, leucine aminopeptidase, acid phosphatase, beta-glucosidase, and phenol oxidase. N-acetyl glucosaminidase, leucine aminopeptidase, acid phosphatase, and beta-glucosidase were measured by fluorometric quantification and phenol oxidase was quantified by absorption. We used 4-methylumbelliferone- and 7-amino-4-methylcoumarin-labeled substrates (200 μm), and L-3,4-dihydroxyphenylalanine (25 mm) substrate to provide quantifiable fluorescence and color

for quantification of oxidation.[26,27] Soil slurries were prepared from 5 g fresh soil in 150 ml sodium bicarbonate buffer (50 mm, pH 7) and homogenized with an immersion blender for 1 min. Hydrolytic enzyme assays were conducted in black 96-well microplates and oxidative assays were carried out in transparent-bottom 96-well microplates. Standard curves were made for each soil sample (soil slurry+4-methylumbelliferone or 7-amino-4-methylcoumarin standard of 0, 2.5, 5, 10, 25, 50 μm). A 200 μl volume of soil slurry and 50 μl of 4-methylumbelliferone or 7-amino-4-methylcoumarin standards were added into wells of standard plate, and 200 μl of soil slurry and 50 μl of the labeled substrate into wells of substrate plate. Plates were incubated in the dark at 25 °C for 3 h and fluorescence was measured immediately after removal from the incubator with a BioTek Synergy HT microplate reader (BioTek Industries, Inc., Winooski, VT, USA) (ex: 365 nm, em: 450 nm). The oxidative enzyme plate contained a buffer blank (250 μl buffer), a L-3,4-dihydroxyphenylalanine blank (200 ul buffer+50 μl L-3,4-dihydroxyphenylalanine), sample blank (200 μl slurry+50 μl buffer) and the sample wells (200 μl slurry+50 μl L-3,4-dihydroxyphenylalanine). Oxidative plates were incubated in the dark at 25 °C for 3 h and absorbance was measured at 460 nm with the BioTek microplate reader. Potential activities were calculated from equations based on previous work.[26,28]

Microbiome 16 S rRNA gene sequencing

Soil DNA was extracted from frozen samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Approximately 0.15 g of soil from each sample was used for isolation of soil DNA. Quantification was performed with the standard dsDNA quantification protocol for Picogreen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All pipetting for DNA extraction was conducted with an Eppendorf epMotion 5075 pipetting robot (Eppendorf AG, Hamburg, Germany). We amplified 16 S rRNA gene sequences in duplicate from the extracted DNA. PCR primers used are described in Caporaso et al. (2012) that target the bacterial/archaeal 16 S rRNA gene variable region 4 (515 F/806 R) for downstream paired-end Illumina (Illumina, Inc., San Diego, CA, USA) barcoded sequencing.[29] Amplicons were quantified with Picogreen and 200ng of each sample were pooled and purified with the desalting protocol of the Qiagen QiaQuick spin filter purification kit (QIAGEN Inc., Valencia, CA, USA). Amplicon pool was submitted to the Cornell Life Sciences Sequencing Core with the custom sequencing primers detailed in Caporaso et al. (2012).

Statistics

Plant trait data were analyzed by nominal logistic regression using JMP Pro 11 (SAS Institute Inc., Cary, NY, United States). Significance between groups was determined by chi-squared distribution at an α-level of 0.05. The drought tolerance groups were established by assigning a score of 1 or -1 to the samples falling within the upper and lower quartile of DGCI values. All others received a score of 0. This was done on three distributions: DGCI at peak drought, DGCI weekly increase during recovery, and DGCI at the end of recovery. These three scores were then summed to create a 7-point (-3 to +3) scoring system for relative drought tolerance in our experimental system. Scores of -3 and -2 were categorized as low tolerance, -1, 0, and 1 were categorized as medium tolerance, and 2 and 3 were categorized as high tolerance.

For 16S rRNA gene sequence analysis, paired-end reads were truncated at the first low-quality base and quality filtered to remove those with an average quality score below 25, fewer than 200nt, ambiguous bases, primer mismatches, erroneous barcodes, and homopolymer runs exceeding six bases. Paired-end reads were joined and then demultiplexed within the QIIME software package (Qiime.org).[30] Operational taxonomic units (OTUs) were picked with uclust against the latest greengenes database clustered at 97% similarity (species-level).[31] Sequences with sequence identity below 69% and sequences matching plant chloroplast or mitochondrial 16S rRNA genes were filtered from the dataset. The smallest number of sequences belonging to any sample was 1000. This value was used to rarify all samples to an even number of input sequences for analysis requiring even sample sizes for robust results. Plots of alpha diversity metrics across rarefaction levels confirm that little community information is gained through inclusion of additional reads. Beta diversity measures (between-sample diversity) were computed with weighted UniFrac and the resulting distance matrix was used to create the principal coordinates plot.[32,33] Log2-fold-change and significance of taxa shifts were computed using the DESeq2 method.[34] The DESeq2 inputs for drought tolerance associated shifts were samples of the high- or low- tolerance group compared to the medium tolerance group to identify taxa shifts associated with an increase or decrease in drought tolerance from the experimental population mean. The DESeq2 inputs for experiment phase associated shifts were Initial samples compared to each Drought and Recovery samples.

Heatmap and ternary plots were created in R. The heatmap was generated with the gplots package from the log relative abundance of key taxa as determined by DESeq2 Bonferroni corrected significance. A value one order of magnitude smaller than the smallest relative

abundance observed was added to zero values prior to log transforming. Ternary plots were created from relative abundances associated with sample categories (experiment phase, drought tolerance).

Results

Soil microbial community analysis revealed strong effects of experiment phase (Initial, Drought, Recovery) on community composition, as shown by principal coordinates analysis generated from weighted UniFrac distances (Figure 1A). However, there appear to be no differences in community composition if the same principal coordinates plot is grouped by drought tolerance (Figure 1B). Very few OTUs are uniquely associated with the drought tolerance groups, which illustrates the large shared microbiome at the center of the plot (Figure 2A). The Initial and Drought phases show similarities in taxa structure, while the Recovery phase contains a lower diversity set of uniquely-associated taxa (Figure 2B).

Experiment phase exhibited the largest effect on microbial community composition. The log relative abundance of the key taxa separated by experiment phase and sorted by drought tolerance levels (Figure 3). The phylum, class, or order of the OTU, based on how closely the phylogeny of the OTU could be assigned, is provided in Supplementary Figure 1. Supplementary Table 1 provides a list of key OTUs, log-2-fold change in relative abundance, and assigned taxonomy between pre-drought, drought, and recovery.

Despite the similarity in community structure between drought tolerance levels, there are differences in a handful of key taxa, all within the Phyla Proteobacteria and Bacteroidetes. DESeq2 reveals significant differences in only two OTUs associated with the high drought tolerance group. These are relative increases in the OTUs assigned to orders Sphingobacteriales and Burkholderiales (f_Burkholderiaceae, g_Burholderia). The low drought tolerance group was characterized by 6 significant taxa shifts. These include decreases in OTUs assigned to orders Opitutales, Ellin329, and Enterobacteriales, and increases in Burkholderiales (f_Oxalobacteriacea, f_Comamonadaceae) and Flavobacteriales.

More evident than the few significant differences in bacterial community composition by drought tolerance, were the differences in potential extracellular enzyme activity and dark septate endophyte infection rates. DSE infection rate is lower in the high-tolerance genotypes

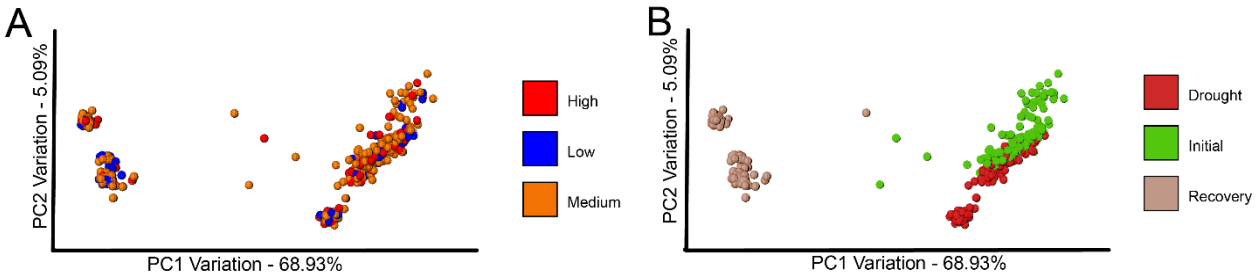


Figure 1. Principal Coordinates Analysis of Unweighted Unifrac Distances

Principal coordinates plot of unweighted unifrac distance matrix illustrates patterns between sample groups. Unweighted UniFrac distances are insensitive to relative abundance of observed OTUs and reveal patterns and differences in the presence/absence of taxa. A) Sample ordination is colored by drought tolerance group and shows no distinct pattern or separation of points by drought tolerance. B) The same ordination from (A), colored by experiment phase (Initial, Drought, and Recovery). Initial and Drought communities are similar, but distinct, and appear to drive the majority of taxa shifts. A large change in the Recovery community as compared to the other phases appears to be responsible for the large percent variation explained by PC1. Samples were rarefied to an even sampling depth of 1000 seqs per sample. Percentages on each axis represent the percent variation explained by each of the PCs.

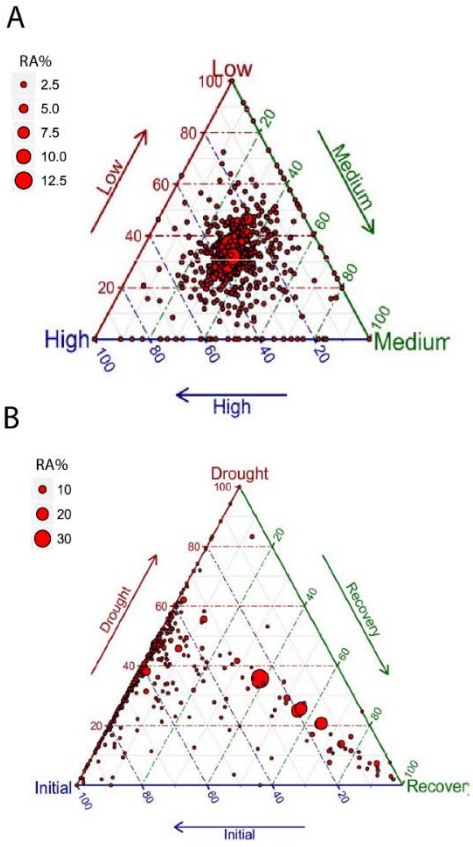


Figure 2. Ternary Plots of Taxa Associations

A) Ternary plot of OTUs showing the percent of each OTU's observations present in each drought tolerance group (Low, Medium, High). For example, a point's position within the '0.8' triangle at the 'High' corner of the ternary plot indicates that 80% of all observations of that OTU occur within the High-tolerance group. The vast majority of OTUs are common to all three tolerance groups. Very few points associate exclusively with any individual tolerance groups. There are several OTUs that are shared between Low and Medium and High and Medium groups, but only two shared between High and Low groups. The size of the plotted points corresponds to relative abundance of the OTU. However, due to the number of small OTU groups, patterns in relative abundance are difficult to resolve. **B)** Ternary plot of OTUs showing the percent of each OTU's observations present in each experiment phase (Initial, Drought, Recovery). Clustering of OTU points shows largely similar Initial and Drought communities, but a unique, low diversity Recovery community. Compartments of the dotted grids correspond to 20% increments.

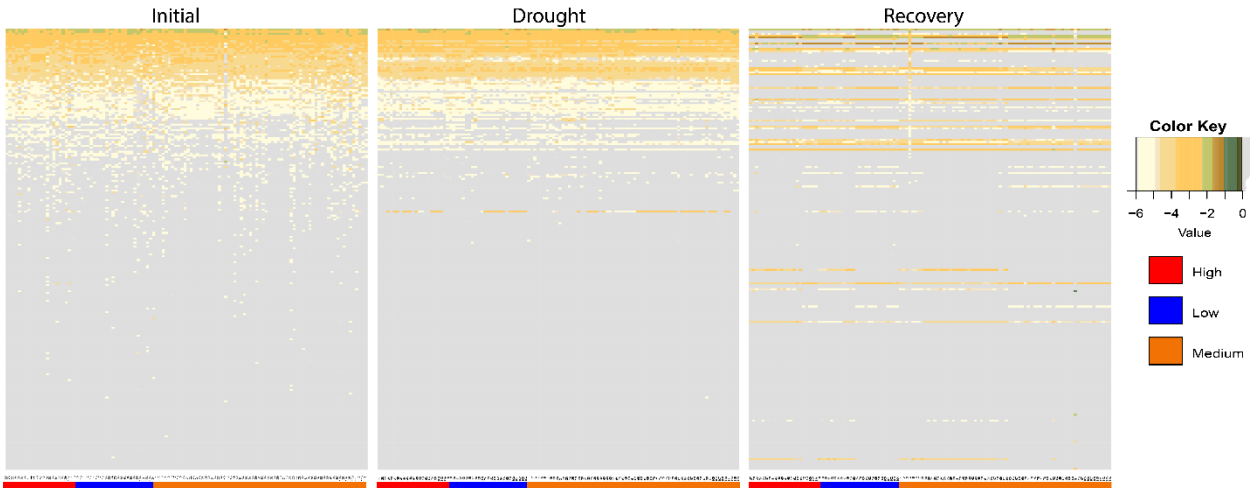


Figure 3. Heatmap of OTU Abundance by Experiment Phase and Tolerance Group

Heatmap of log relative abundance of key taxa identified by DESeq2. Significance of taxa shifts determined at alpha-level 0.05 from false discovery rate corrected p-values. A value one order of magnitude smaller than the smallest relative abundance observed was added to zero values prior to log transforming. Color bars at the bottom of the figure indicate the tolerance group. (Low: Blue, Medium: Orange, High: Red,). Each of the three heatmap sections correspond to the experiment phase: Initial, Drought, Recovery. OTUs are clustered by average abundance in the Initial phase to highlight differences between phases.

(Arcsine-transformed DSE% 0.196 ± 0.02 SE) than either the low- and medium- drought tolerance groups (Arcsine-transformed DSE% 0.23 ± 0.01 SE) (Figure 4). Of the assayed extracellular enzymes, acid phosphatase (AP) and phenol oxidase (PO) activity were significantly associated with drought tolerance. Higher AP and PO activity at peak drought corresponded to better tolerance overall (Figure 5).

Discussion

In this study, we investigated microbial community composition and activity in tall fescue rhizospheres under and during recovery from drought stress. Previous studies have shown extracellular enzyme changes in response to drought are variable.[35–37] In this study, greater drought tolerance corresponded to increases in acid phosphatase and phenol oxidase activity during the drought phase. The shift toward greater production of these enzymes upon drought conditions may be related to mineralization of soil phosphorus or nitrogen as a means to cope with drought and hasten recovery.

The production of extracellular enzymes provides a major mechanism by which microorganisms gain access to limiting nutrients bound in soil organic matter. Groups of microorganisms can produce extracellular enzymes in response to nitrogen- or phosphorus-limiting conditions by depolymerizing soil organic matter for uptake of released nutrients.[38–42] Bacteria and fungi both produce extracellular enzymes, and the increase in acid phosphatase and phenol oxidase activity may signal a role in drought tolerance or recovery. Plants are also able to produce phosphatases, but their contribution to the acid phosphatase activity presented here is unknown.

Originally, we expected differences in microbiome function to be accompanied by shifts in microbial community composition and DSE infection rates. However, changes in extracellular enzyme activity by drought tolerance were not accompanied by changes in the soil bacterial community. This aligns with the ecological concept of functional redundancy, or the idea that multiple organisms in a community can fulfill the same functional role in the ecosystem.[43] An ecosystem with overlapping functional groups or multi-functional taxa can exhibit plasticity in function in the absence of changes in composition. The composition of the soil bacterial community was driven primarily by the different sampling phases (Initial or pre-drought, Drought, Recovery). The microbial community responded to drought with slight shifts in overall composition, a decrease in diversity, and significant decreases in OTUs of phyla Actinobacteria,

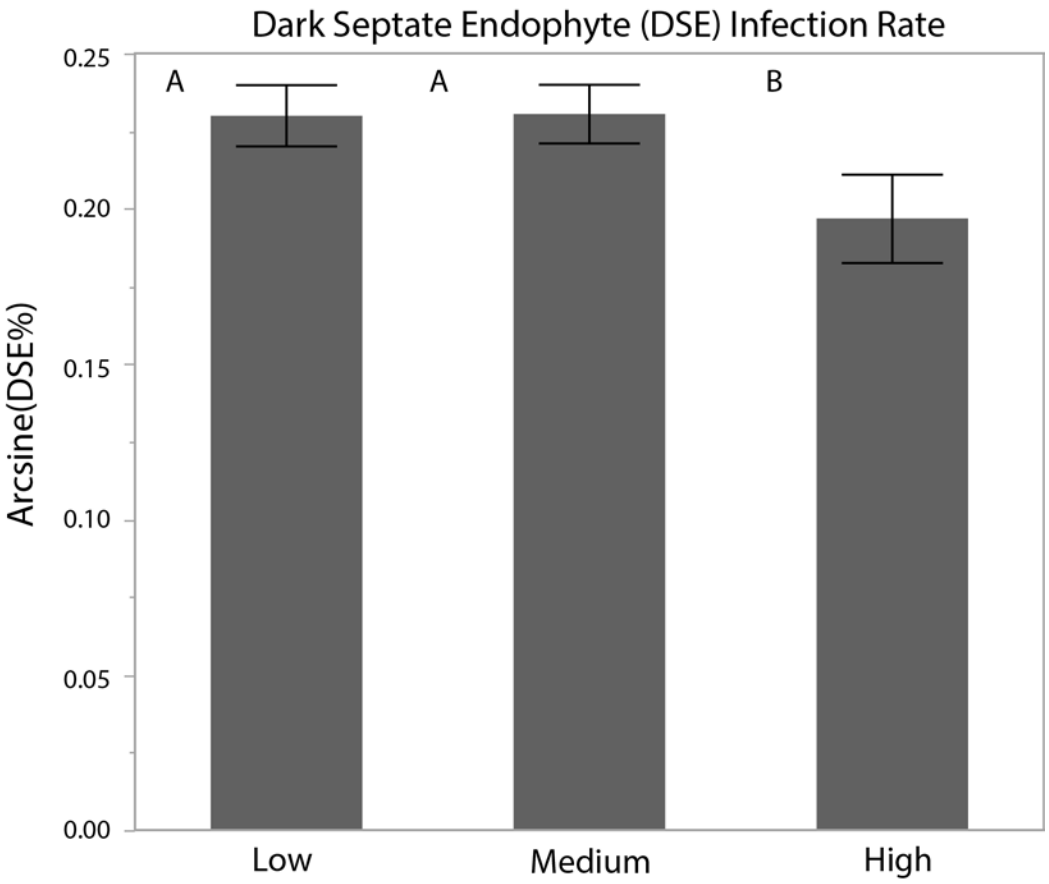


Figure 4. Arcsine-transformed Dark Septate Endophyte Infection-rates by Tolerance Group

Histogram of Arcsine-transformed dark septate fungal endophyte infection-rates between drought tolerance groups prior to drought. Infection rates were determined by dividing the number of infected root cells by the total number of grid cells observed through microscopy. Connecting letters report indicates significance at alpha-level 0.05. Error bars are standard error of the mean.

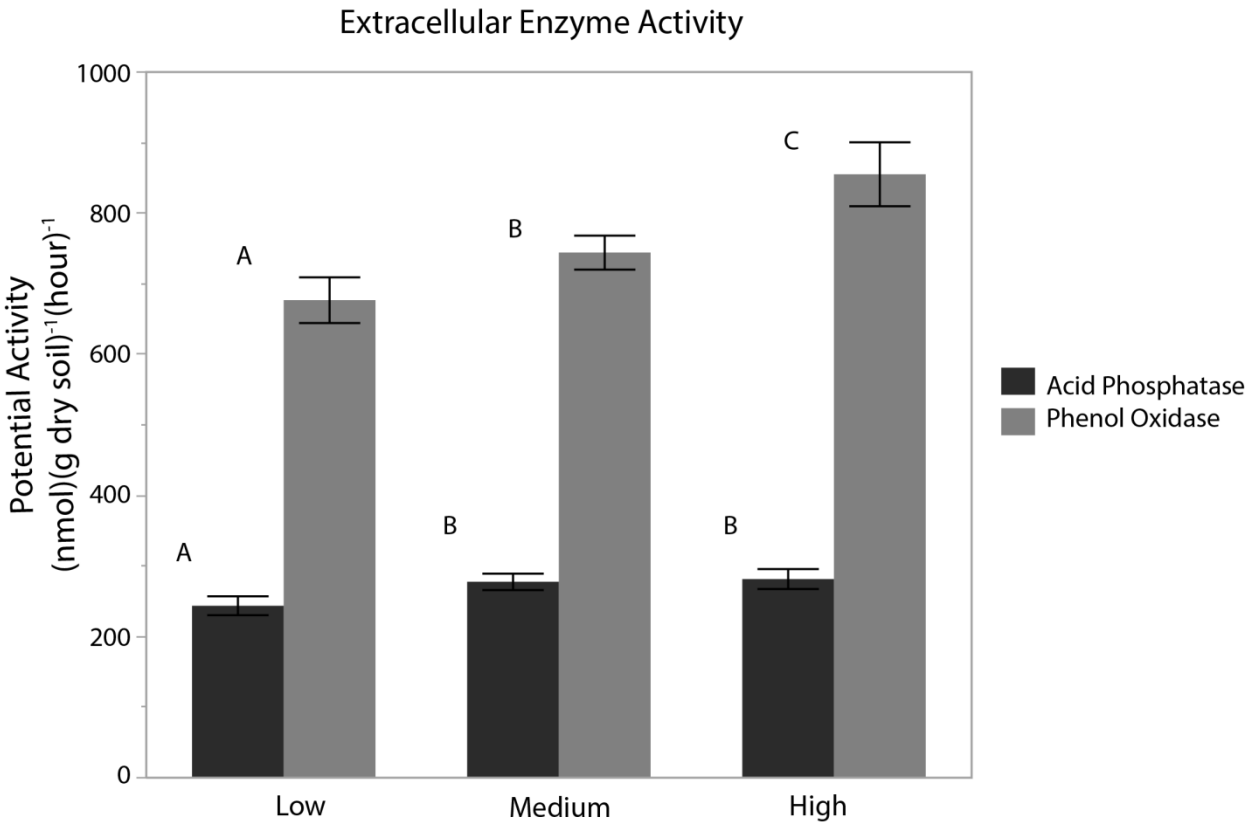


Figure 5. Extracellular Enzyme Activity by Tolerance Group

Histogram of acid phosphatase and phenol oxidase extracellular enzyme potential activities. "Potential" in this context refers to the *in vitro* nature of the assay conditions as opposed to *in situ* values. Connecting letters reports indicate significance at alpha-level 0.05 and are specific to acid phosphatase and phenol oxidase data series next to which they appear. Error bars are standard error of the mean.

Bacteroidetes, and Proteobacteria and increases in Crenarchaeota, Verrucomicrobia, Nitrospirae, and WS3. Overall effects of drought on community composition are in agreement with previous studies, with a decrease in diversity and a difference in community composition.[37,44–46] It should be noted however, that none of these studies use 16s rRNA gene sequencing as a method of profiling the soil microbial community. Recovery was characterized by shifts consistent with a strong selective pressure toward fast-growing and/or drought tolerant taxa; and a large decrease in diversity, likely indicating a dominance of drought-tolerant and rapidly recovering taxa. These observations agree with previous studies' presentation of soil microbial community sensitivity to drought and recovery.[44,46,47] Significant key taxa relative abundances greater than 2 (log-2-fold) different from initial relative abundance reveal overall increases in OTUs within phyla Crenarchaeota, Acidobacteria, Firmicutes, Chloroflexi, Verrucomicrobia, Nitrospirae, Gemmatimonadetes, Actinobacteria, and WS3 and decreases in OTUs within phyla WS2 and FBP. Increases in Crenarchaeota, Verrucomicrobia, and Nitrospirae mostly correspond to those observed during drought. Phyla Proteobacteria and Bacteroidetes were the most abundant taxa and exhibited large shifts in taxa presence and abundance. For the most part, these shifts consisted of increases and decreases in OTUs assigned to similar or identical taxa, indicating shifts at too fine a resolution for us to interpret. However, at the order level, Proteobacteria showed clear decreases in Rhodospirillales, Ellin 329, and Caulobacterales; Bacteroidetes exhibited a decrease in Sphingobacteriales.

Despite no major differences in community composition by drought tolerance, DESeq2 revealed shifts in a handful of operational taxonomic units (OTUs) corresponding to low or high drought tolerance. The high drought tolerance group was associated with increases in the orders Sphingobacteriales and Burkholderiales (genus Burkholderia). Previous studies have shown shifts in Sphingobacteriales in response to salinity-induced water stress[48] and the genus Burkholderia to be associated with drought tolerance.[1] The low drought tolerance group exhibited shifts in six key OTUs. Ellin 329, common in rhizosphere samples, Opitutales, a common soil and marine gram-negative, cell wall-less taxon, and Enterobacteriales, the order containing the Erwinia plant pathogens were all lower in relative abundance when compared to the medium tolerance group, while families Comamonadacea, and Oxalobacteriacea and order Flavobacteriales were all higher in relative abundance. Oxalobacteria is a common rhizosphere

taxon,[49] Flavobacteria has been associated with salinity-induced water stress,[48] and Comamonadaceae contains denitrifying taxa.[50]

We did observe changes in DSE infection rates between drought tolerance groups, but in this case, a lower presence of DSE corresponded with higher drought tolerance. DSE are more prevalent in semiarid grasslands than, and functionally similar to, AMF in their ability to increase plant growth and abiotic stress tolerance.[13] Based on previous studies, greater association with DSE was expected to increase plant drought tolerance (Barrow, 2003; Porras-Alfaro *et al.*, 2008). The lower colonization rates in the high drought tolerance group suggests a fitness cost associated with heavier DSE infection during drought; however, there are no published studies we are aware of that support this idea. In addition, most symbiotic relationships are mutually punitive; each participant may withhold their contribution to the mutualism in the absence of the other participant's contribution (Egas & Riedl, 2008). These findings suggest that decreased association with DSE is likely not causative in higher drought tolerance.

The increase in extracellular enzyme activity is also contrary to expectations given the decrease in DSE. DSE are capable of degrading a wide variety of substrates through extracellular enzyme production (Caldwell *et al.*, 2000). Previous observation has revealed maintenance of plant carbon allocation to the fungal community during drought at the expense of the bacterial community.[55] Correspondingly, we would expect higher enzyme activities in plots with higher DSE infection rates. Instead, we observed the opposite, which suggests that the increase in extracellular enzyme activity is attributable to the plant, bacterial, or un-profiled fungal communities.

Plant contribution to observed acid phosphatase activity could not be separated from microbially-derived activity. However, contributions of plant-derived phosphatase are limited to the root-soil boundary by low solubility; an obstacle even further exacerbated under drought conditions.[56] Furthermore, given observations of organic acid accumulation and exudation in response to drought stress and increased turnover of root tissue in low tolerance genotypes due to death, we would expect the low tolerance group to show increased activity if the plant were playing a major role.[57,58]

These results suggest that soil bacterial community activity may play a role in tall fescue tolerance to drought despite little to no differences in microbial composition. This highlights the

value of data regarding microbiome activity, because composition does not necessarily reflect changes in function. Specifically, the role of soil extracellular enzymes should be given more attention in studies on plant drought tolerance and recovery.

Conclusion

Here, we have shown that, even in the absence of changes in microbiome composition, variation in microbiome function plays a key role in plant genotype-specific responses to drought stress. This highlights the need for inclusion of methods that capture functional microbiome traits in addition to 16S rRNA gene community composition. The trends evident in the DSE infection rates support the need for further study of how root endophytes alter drought responses in plant hosts.

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CONCLUSION

The dissertation work contributes to the expanding body of research regarding the plant rhizosphere microbiome. Specifically, it puts forth the idea of “breeding” rhizosphere microbiomes for the purpose of modifying plant host traits. Ecosystem selection and cultivation are presented here as a means of enhancing and characterizing microbiome-mediated effects on plant growth and development. These studies demonstrate the ability to select for rhizosphere microbiomes that alter soil processes, plant flowering phenology, biomass accumulation, and partitioning. Furthermore, these effects are reproduced in novel plant hosts. Cultivated subsets of selected microbiomes exhibited similar primary plant response (flowering time), but revealed modulation of the secondary response (leaf biomass). This presents selection and cultivation as an experimental platform for refining functional microbiomes to facilitate mechanism-level understanding of their interaction with plants. The parallel investigation of rhizosphere microbiome function between plant genotypes and the response of the microbiome to selective pressure is a first step in gauging the potential of microbial components in traditional plant breeding programs. Together, these studies suggest that harnessing the plant microbiome through artificial selection, cultivation, and parallel plant breeding is a potential tool for addressing key agronomic and environmental concerns