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Investigating the Causes and Consequences of Drought-Induced Endophytic Actinobacteria Enrichment

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Investigating the Causes and Consequences of Drought-Induced Endophytic *Actinobacteria* Enrichment

By

Tuesday B. Simmons

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Committee in charge:

Professor Devin Coleman-Derr, Co-Chair
Professor Adam Arkin, Co-Chair
Professor Britt Koskella
Professor Matthew Traxler

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Abstract

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Since the advent of agriculture, humans have been working to improve the growth of crop plants. Historically, a major focus of research has been plant genetics and using traditional breeding methods to improve crop yields and adapt crops to demanding environmental conditions. More recently, the role of microorganisms living in and on plants, referred to as the plant microbiome, has been a target of investigation for its role in plant growth and development. Many factors have been found to influence the plant microbiome: host genetics, geography, agricultural practices, and soil chemistry, to name a few. One other significant factor is water availability; when plants are subjected to drought stress, the root microbiome displays a pattern of enrichment of *Actinobacteria*. The work presented herein seeks to understand the mechanism behind the enrichment of this phylum, as well as the effect of the microbial community shift on the growth of the host plant.

This research begins by investigating the effect of drought stress on the root microbiome of different species of millets and honing in on the spatial distribution of enriched *Actinobacteria* within the root endosphere. By using 16S rRNA amplicon sequencing to explore the bacterial communities, we established that different degrees of drought are correlated with the level of *Actinobacteria* enrichment in four species of millet. To narrow down the plant signal that might be responsible for the enrichment, we asked whether this pattern was dependent upon root age. We found that the enrichment occurs along the length of plant roots, which suggests that *Actinobacteria* are proliferating within roots, since the majority of colonization has been shown to occur in the youngest part of the roots (at the root tip). Additionally, to determine whether the plant signal driving enrichment was one that is localized to the area of drought stress or if it is spread throughout the root system, we set up a split-pot experiment to expose only a portion of a plant's roots to drought. In this case, only the drought-stressed portion shows enrichment, suggesting the driving mechanism is not spread throughout the root system. Finally, we sought to establish whether the mechanism for enrichment was death of the stressed roots selecting for saprophytic microbes, which include *Actinobacteria*. By profiling living and dead roots from the same host plant grown in a greenhouse setting, we show that *Actinobacteria* are depleted in dead

root tissue, suggesting saprophytic activity is not the driving cause behind the shift in the microbial community structure. Overall, these results show that enrichment of *Actinobacteria* in drought-stressed roots is dependent on localized drought responses but not root age or death.

To investigate how *Actinobacteria* impact the growth of the host plant, the next goal was to develop a diverse strain collection by isolating bacteria from the roots of drought-stressed sorghum. For downstream experiments, it is best, in our case, to use microbes isolated from the environmental background that is being studied, rather than obtaining isolates from a strain collection with an unknown or different background. Using two isolation workflows, we collected nearly 2000 strains of bacteria. We showed that, while it may be beneficial in some cases to build an isolate library by selecting colonies from agar plates, using high-throughput isolation technologies can yield a much larger library of a comparable diversity in much less time. Additionally, we show that selection of media type is important when building a specialized library, and differs between the two isolation workflows.

Finally, using the specialized strain collection, we investigated the efficacy of Synthetic Communities (SynComs) containing *Actinobacteria* on improving the growth of plants in an otherwise sterile environment. We used a co-occurrence network built using SparCC to hypothesize interactions between bacteria in drought-stressed root endophyte communities, and from this network selected closely-related strains from our collection to compile into SynComs. Seven SynComs were designed: five of them ranged from 100% to 0% *Actinobacteria* in intervals of 25%, one contained only Gram-negative bacteria, and one contained only *Streptomyces*, a known plant growth promoting taxa. After applying these communities to plants, we found that they were able to colonize the roots and persist over time. Additionally, we found the SynCom containing only *Streptomyces* to be most beneficial to plant growth, suggesting that interspecies interactions within the 100% *Actinobacteria* (which consisted of the same *Streptomyces* plus additional *Actinobacteria* genera) may inhibit the plant growth promoting activity of certain microbes.

Altogether, the studies presented herein contribute to our understanding of the root microbiome during drought and the ecological principles governing the microbial communities of the endosphere. We provide clues as to what host plant mechanism may be driving the enrichment of *Actinobacteria* during drought, and begin to uncover the community dynamics that could lead to the design of a synthetic community that would effectively protect a host plant from drought stress.

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Dedications

For my parents, James and Karla Moats, my first and greatest teachers.

Chapter 1: Introduction

1.1 Plant root microbiome

Understanding the relationship between microorganisms and the plants they live with is recognized as a critical component of agriculture (Parnell et al. 2016). We know that microbial soil communities change along gradients of different chemical and physical parameters (Brockett, Prescott, and Grayston 2012; Zheng et al. 2019; Cookson et al. 2007; Maestre et al. 2015; Lauber et al. 2009; Zhou et al. 2016; Leff et al. 2015; Whitman et al. 2018), so it is logical that we would observe differences between the bulk soil, the substrate outside the zone of plant root exudates, and the rhizosphere, the layer of soil into which plants exude carbohydrates and other organic material (Chaudhary et al. 2012; Wieland, Neumann, and Backhaus 2001; Bulgarelli et al. 2013). Extending on this, it is also logical that the microbial community living within plant tissue, the endosphere, is distinct from those living in the surrounding rhizosphere (Coleman-Derr et al. 2016; Santos-Medellín et al. 2017). Much like the human microbiome, different plant tissues contain different microbial communities: the root community is different from the stem, leaf, and seed communities (Compant et al. 2019; Human Microbiome Project Consortium 2012). Understanding the root community is arguably the most important, as subsets of these microbes can assist plants in assimilating essential nutrients and fight off pathogens that otherwise infect the roots (Bulgarelli et al. 2013; Osorio Vega 2007; Schlatter et al. 2017). We know that there are several compounding factors that shift the structure of the root microbial community including geography, and abiotic factors, as well as host genetics, which includes production of metabolites that feed root associated microbes (Fitzpatrick et al. 2018; Xu et al. 2018; J. A. Edwards et al. 2018; J. Edwards et al. 2015).

Much like selective media used in the laboratory, plants synthesize a distinct suite of metabolites that may change with age and environmental conditions (Iwanycki Ahlstrand et al. 2018); root-derived metabolites may help select which bacteria from the bulk soil are able to colonize the rhizosphere and the root endosphere (Zhalnina et al. 2018; Xu and Coleman-Derr 2019). Molecules in root exudate include: carbohydrates, amino acids, organic acids, lipids, coumarins, flavonoids, and polysaccharide mucilage (Reinhold-Hurek et al. 2015). In addition to secretions, a lot of organic carbon is deposited in the rhizosphere from plant root cap border cells that have been sloughed off during growth (Bais et al. 2006). The utilization of these metabolites by microorganisms has been shown using stable isotope probing experiments. When $^{13}CO_2$ is given to plants and fixed through photosynthesis, the labelled carbon is found in non-photosynthetic microbes (Haichar et al. 2008; Lu et al. 2006). Similar experiments have been done to show that microbes also take up exuded amino acids (Moe 2013). Since these exudates have the potential to select for or against microbial symbionts, the host plant has a fair amount of selective control, and it would be evolutionarily favorable for the plant to select for beneficial partners.

Some bacteria that colonize the root microbiome are classified as Plant Growth Promoting Bacteria (PGPB) as a result of their ability to enhance the growth of their host plant through a variety of mechanisms (Souza, Ambrosini, and Passaglia 2015). These can also be

referred to as PGPR (Plant Growth Promoting Rhizobacteria) or included in a term that also encompasses eukaryotes and archaea: PGPM (Plant Growth Promoting Microorganisms). PGPB facilitate plant growth using a diverse range of mechanisms. The most well-studied PGPB function is perhaps the assistance of nutrient acquisition through nitrogen fixation. While some nitrogen-fixing bacteria live in specialized structures such as the root nodules of legumes or specialized mucilage on maize roots, they also exist as free-living organisms within the rhizosphere (Stougaard 2000; Van Deynze et al. 2018; Smercina et al. 2019). Bacteria can also be involved in the solubilization and uptake of phosphate (Chen et al. 2006; Rodríguez et al. 2006). Additionally, some PGPB are able to synthesize and alter plant growth hormones such as ethylene and auxin precursors (Glick 2012). There are other PGPB that assist in plant growth indirectly - through interference with pathogens as a method of biocontrol (Mendes et al. 2011). As researchers have uncovered microbial traits that enhance plant phenotypes, these microorganisms have become a target for agricultural applications.

Humans have been inoculating soil with PGPB for millennia, as an extra component of the original fertilizer - compost (Diaz and de Bertoldi 2007). The first bioinoculant "nitrogin" was patented in 1896 by Friedrich Nobbe and Lorenz Hiltner and contained bacteria that triggered the formation of nodules on legumes (Nobbe and Hiltner 1896). This was 17 years before nitrogen fertilizer would be mass produced using the Haber-Bosch process in 1913 (Kissel 2014). As we become more aware of how detrimental excess agricultural inputs can be on the environment, farmers and agricultural scientists have been working to minimize the use of chemical fertilizers and pesticides (Wu et al. 2018). One way to do that is to supplement or even replace these chemicals with microorganisms that can do the same job. Inoculating with PGPM that can act as pesticides, protect against pathogens, and help assimilate nutrients can minimize the chemical inputs needed to maintain a high crop yield. Unfortunately, the primary obstacles to adopting bioinoculants in agriculture are systemic (risk-averse customers, a lack of infrastructure, and lack of awareness, to name a few) (Marrone 2007). Scientifically, as we move forward in the application of this field, we should move toward understanding the ecology of PGPM. While it is important to understand the molecular mechanisms by which they benefit their plant hosts, we also need to understand the role PGPM play in their community: how well they establish and persist, how they prevent unintended functional changes in the community, and what impacts PGPM might have on "macro" species other than the plant host are all important points.

1.2 Drought

A system experiences drought when there is a long-term deficiency of precipitation that results in a shortage of water. Factors that contribute to drought include reduced precipitation, soil physical properties (health and stability), and increased evaporation; these reduce the amount of water available to crops, which leads to lower agricultural production (Gornall et al. 2010). It has been estimated that by 2050, yield reductions in major crops (maize, barley, rice, and wheat) that can be attributed to drought will increase by more than 50 percent (Li et al. 2009). Since this is an issue that will only continue to worsen as climate change drives the increasing frequency and severity of drought (IPCC 2014), substantial effort has been made to understand the effects

of drought on crops at a molecular level, and to establish new methods of improving crop yields when water is a limiting resource.

The physiological and morphological response of plants to drought has been studied extensively and has become even more important in recent years. In addition to decreasing overall plant growth, root architecture changes in eudicots during drought by decreasing the angle, length, and number of lateral roots, and increasing the length of primary roots (Koevoets et al. 2016). Monocots under drought stress will switch from growing crown-derived to primary root-derived branches, resulting in roots that reach deeper into the soil (Rellán-Álvarez, Lobet, and Dinneny 2016). At a molecular level, plants increase generation of reactive oxygen species (ROS) and osmolytes such as proline, sugars, and proteins that help to relieve the osmotic stress on cells (Apel and Hirt 2004; Serraj and Sinclair 2002; Kosová, Vítámvás, and Prášil 2014). Additionally, drought increases the production of ethylene, which inhibits growth (Dubois, Van den Broeck, and Inzé 2018). Arguably one of the most important hormones involved in plant drought response is abscisic acid (ABA), which is responsible for regulating stomatal closure to reduce water loss as well as signal transduction.

Of recent interest has been the impact of drought on the root microbiome. Logically, if drought is causing a change in plant physiology, the plant will produce a different suite of metabolites, which would in turn change the community of microorganisms suited for that environment (Michaletti et al. 2018; Z. Kang et al. 2019). It is possible that plants have evolved such that this change in metabolite profile will select for microbial symbionts that benefit plant growth during drought. Indeed recent work has shown that drought has a significant impact on the structure of the root microbiome (Xu et al. 2018; Naylor et al. 2017; Fitzpatrick et al. 2018; Santos-Medellín et al. 2017). Specifically, we and other groups have observed significant enrichment of monoderm bacteria (primarily *Actinobacteria*, *Firmicutes* to a lesser degree, and a few *Chloroflexi*) within the root endosphere that does not occur in the surrounding soil during drought stress (Naylor et al. 2017; Santos-Medellín et al. 2017; J. A. Edwards et al. 2018; Fitzpatrick et al. 2018; Xu et al. 2018). While many studies have focused on a single plant host, a few have looked at multiple host species grown in the same environment and showed that *Actinobacteria* were enriched across hosts (Naylor et al. 2017; Fitzpatrick et al. 2018). Additionally, when studying the root microbiome of desert plants, Marasco et al (2018) has shown *Actinobacteria* dominate. This has led us to ask - are *Actinobacteria* beneficial for plant growth during drought?

Within the *Actinobacteria* lineage, the genus *Streptomyces* has been particularly well characterized. Many species of *Streptomyces* have been shown to benefit host fitness (Vurukonda, Giovanardi, and Stefani 2018; Worsley et al. 2020; Viaene et al. 2016). It has also been shown that *Streptomyces* are able to benefit plant growth during times of drought (Xu et al. 2018; Yandigeri et al. 2012). These species have several genomic clues to suggest ways in which they might be beneficial. First, they are perhaps most famous for the wide array of antibiotics and antifungals they are able to produce, which could protect their host from pathogens (Millard and Taylor 1927; Newitt et al. 2019; Suárez-Moreno et al. 2019). Second, they have genes to modulate phytohormones, such as ACC deaminase and IAA (Worsley et al. 2020). Finally, members of this species are able to assist in nutrient acquisition through nitrogen fixation,

phosphate solubilization, and iron uptake (Vurukonda, Giovanardi, and Stefani 2018). Considering the genomic potential of *Streptomyces* and other *Actinobacteria*, many groups are investigating the application of these and other species to be applied as PGPB in agricultural settings. Large companies such as Bayer, FMC, Corteva Agriscience, and Syngenta are investing in the production of biologicals as a supplement to, and potentially an eventual replacement for chemical fertilizers and pesticides.

While most research has been focused on pathogen and nutrient stress, less is known about how *Actinobacteria* can be beneficial during abiotic stress such as drought. Additionally, while current academic and industrial studies have focused on individual *Streptomyces*, the work herein investigates how *Streptomyces* species function as part of synthetic communities designed to improve plant growth during drought.

1.3 Plant models for drought stress in agriculture

When selecting a study organism to investigate the relationship between plant hosts and their root microbiome during drought, we wanted a domesticated crop that has some drought tolerance and is economically important. We predicted that drought-tolerant plants would harbor the largest number of PGPB. In contrast, grains such as rice, wheat, and maize are the most frequently grown staple crops on earth (Awika 2011), however none of these are able to withstand drought well (Daryanto, Wang, and Jacinthe 2016; X. Yang et al. 2019). A close relative of these crops (within the same family Poaceae) that is known to be drought-hardy is *Sorghum bicolor*, or colloquially known as sorghum. Rated by the US Grains Council as one of the most drought tolerant cereal crops currently grown (Ananda, Vadlani, and Prasad 2011), sorghum is widely grown in semi-arid regions such as sub-Saharan Africa, where it was domesticated. Additionally, it has economic importance as both a livestock feed and for ethanol production (Paterson et al. 2009).

Sorghum is closely related to a larger group of polyphyletic cereal crops called millets. While not included as part of the group, sorghum is often called “great millet”. As a whole, millet grains provide a primary source of food and livestock feed for hundreds of millions of people in arid regions of Africa and Asia (Patil 2017). Like sorghum, other millet species are known to be drought tolerant. These crops are often grown on marginal lands with sporadic, rain-fed irrigation, which leave them exposed to water stress during periods of drought (Kumar et al. 2018). In the studies herein, we worked with five different members of the Poaceae family: *Sorghum bicolor* and the millets *Setaria italica* (foxtail millet), *Pennisetum glaucum* (pearl millet), *Panicum miliaceum* (proso millet), and *Echinochloa esculenta* (Japanese barnyard millet).

1.4 Studying model microbes: cultivation

In the past 40 years, our ability to study environmental microorganisms without cultivation has skyrocketed through the use of -omics technologies (metagenomics, transcriptomics, metabolomics, etc.) (Pace et al. 1986). These methods have been praised for getting around the issue known as the “great plate count anomaly” - that only a small percentage of microorganisms can be grown on plates in the lab (Staley and Konopka 1985). Through

-omics techniques, we have uncovered driving factors behind the root microbiome, and broad taxonomic patterns have been observed during drought. However, there is a degree of variability at finer taxonomic resolutions, making it difficult to define a "model root microbiome" (Naylor et al. 2017). There is evidence that microbial communities from similar habitats have core functional groups despite taxonomic differences (Estrela et al. 2020; Shade et al. 2012), and these functions can be studied more rigorously with synthetic communities consisting of natural isolates.

After years of learning about the metabolic requirements of microbes through sequencing and computational work, microbiologists are returning to work in the lab with individual isolates to better understand the discoveries made using -omics techniques. Microbiologist Paul Carini has recently stated: "Unculturable is a frame of mind, not a state of microbiology" (Carini 2019). Two developments have contributed to the advancement of working with "unculturable" microorganisms: 1) an improved understanding of the metabolic requirements of environmental microbes, and 2) improved technologies that facilitate high-throughput isolations. While it may still be beneficial in some instances to isolate microbes one at a time using a hand-picking method from plates, there is interest in scaling up the number of microbes that can be isolated at a time. For example, GALT (General Automation Lab Technologies, San Carlos, CA, USA) has developed a system called the Prospector™ that allows for high-throughput isolations from environmental samples. Using this system, a single scientist can isolate thousands of distinct microbes in just a few months, which would otherwise take years of hand-picking from plates. Having a large isolate collection on hand is invaluable for bottom-up approaches to studying the function of different microbiomes. These isolates can be used to validate functional hypotheses derived from -omics studies, application of potential PGPM to gnotobiotic plants, and investigate pairwise and larger-order interactions in the form of synthetic communities.

1.5 Synthetic Communities

We know that there are PGPB that are able to enhance host plant growth as a single inoculum, though most of these experiments are done in limited size containers in growth chambers or greenhouses. When individual isolates are applied to a field system that already has an established and stable soil microbiome, the applied isolate may be unable to successfully invade the existing community and persist in the soil (Shade et al. 2012; van Elsas et al. 2012). For this reason, we suggest the application of stable synthetic communities (SynComs) to soil systems as biofertilizers, biopesticides, and protection from drought stress (Vorholt et al. 2017; Fitzpatrick et al. 2020; Castrillo et al. 2017).

In order to construct the most stable SynComs, we propose three criteria to be followed. First, communities should consist of more than 2-3 members, since higher diversity will increase stability (B. S. Griffiths et al. 2000; Loreau and Hector 2001; Shade et al. 2012). We constructed 16 member communities, since a much larger size becomes difficult to maintain and perform follow-up experiments to determine the role each strain plays in the community. Second, rather than selecting a random group of individual microbes that might be beneficial for the host plant, these communities should be selected carefully by using known or hypothesized interactions

between members of a common root microbiome. Finally, we hypothesize that using isolates derived from the same environment that they will be applied to (in this case, sorghum roots) will maximize stability. Additionally, this would mean that all the isolates have the same environmental history.

By following these criteria, we can design SynComs that are manageable yet representative of natural communities, a major goal when reconstructing microbiomes using a bottom up approach. In our study, we used an existing dataset (Xu et al. 2018) from amplicon sequencing of drought-stressed root endophyte communities to predict interactions between community members using a co-occurrence network. From this, we selected isolates collected from the same host plant that closely matched the ASVs in the network, and applied them as communities to gnotobiotic plants. These communities will allow us to answer ecological questions such as: which isolates may have plant-growth promoting properties only when applied as a community, which community members may be functionally complementary or redundant, and which isolates (or functions) may act as keystone species.

1.6 Concluding introductory remarks

In the chapters herein, I describe three projects designed to investigate the mechanisms behind the enrichment of endophytic *Actinobacteria* in cereal crops and whether the enrichment is beneficial to plant growth. In Chapter 2, I aim to uncover the spatial distribution of enriched *Actinobacteria* during drought in different millet species. To better understand the causes of this phenomenon, I performed a series of experiments in millet plants to explore the roles of drought severity, drought localization, and root development in driving *Actinobacteria* enrichment. It is shown that the degree of drought is correlated with levels of *Actinobacterial* enrichment in four species of millet grown under a water gradient. Additionally, we demonstrate that the observed drought-induced enrichment of *Actinobacteria* occurs along the length of the root, but only in response to local, rather than systemic, water stress. Finally, we demonstrate that *Actinobacteria* are depleted in the dead root tissue of Japanese millet, suggesting saprophytic activity is not the main cause of observed shifts in drought-treated root microbiome structure. Collectively, these results help narrow the list of potential causes of drought-induced *Actinobacterial* enrichment in plant roots by showing that enrichment is dependent upon local perception of drought stress but not root developmental stage or root death.

In Chapter 3, my goal was to develop a diverse bacterial isolate collection that would allow us to investigate interspecies bacterial interactions as well as plant-microbe interactions during drought. I describe the construction of a strain library composed of *Actinobacteria*-enriched bacterial endophytes collected from drought-stressed sorghum roots using two methods. First, using the standard method of hand-picking colonies from agar plates of various media types, we collected about 400 isolates. Next, we performed high-throughput isolations by working with a company, GALT, Inc., to adapt their existing robotics platform for use with plant root material. Using this system, we collected an additional 1500 isolates. The use of these two methods showed that we are able to build a collection of targeted organisms (in our case, of *Actinobacteria*), rather than a broad library. The importance of having a strain collection

on hand for future experiments to investigate the molecular underpinnings of these interactions cannot be understated, and is highlighted explicitly by the research in the final chapter.

Finally in Chapter 4, my aim was to develop a method for constructing stable SynComs and use these communities to better understand the role of *Actinobacteria* in drought-stressed roots. Herein, I describe a study that utilizes the strain collection described in Chapter 3. Using existing data for sorghum root endophyte community structure during drought, I built a co-occurrence network to predict which community members have either positive or negative interactions. From these interactions, I constructed SynComs that range from 100% to 0% *Actinobacteria* using isolates in the existing strain collection. These SynComs were used to test for plant-growth promoting activity of whole communities, and we found that the most beneficial community applied during drought was one in which there were only *Streptomyces*. Future work will determine which members of the SynComs were responsible for benefiting plant growth and will investigate the mechanism by which they do so.

Chapter 2: Investigation of Drought-Induced Spatial Distribution of Enriched Endophytic *Actinobacteria* in Millets

Parts of this chapter have been adapted from the following with permission:
Simmons T, Styer AB, Pierroz G, Gonçalves AP, Pasricha R, Hazra AB, Bubner P and Coleman-Derr D (2020) Drought Drives Spatial Variation in the Millet Root Microbiome. Front. Plant Sci. 11:599. doi: 10.3389/fpls.2020.00599

Author Contributions
PB and AH designed the multi-species field experiment. TS and DC-D designed the greenhouse, lab, and sorghum field experiments. AG and RP performed sample collection and phenotypic measurements for the multi-species experiment. TS performed the sample collection for the remaining experiments, sample preparation, and library preparation. GP performed the microscopy. TS, AS, and DC-D performed the statistical analyses and manuscript preparation.

2.1 Introduction

Drought is a major obstacle to agricultural productivity. It is currently the climate phenomenon with the greatest negative impact on cereal production (Lesk, Rowhani, and Ramankutty 2016), and the severity and frequency of drought is expected to increase in the coming decade (Vicente-Serrano and Lopez-Moreno 2014; Spinoni et al. 2018). As such, it represents one of the largest challenges to food security (Kogan, Guo, and Yang 2019), especially considering the anticipated increases in food production that will be needed to feed the growing world population (Ray et al. 2013). Historically, crop breeding has helped select for drought resistant cultivars, but such efforts are often time and labor intensive (Coleman-Derr and Tringe 2014). For these reasons, development of alternative strategies of protection against drought's negative impacts on crop fitness are needed (Y. Kang, Khan, and Ma 2009; Lesk, Rowhani, and Ramankutty 2016).

Microbially-mediated crop fortification is currently touted as an attractive strategy for mitigating drought stress (Naylor and Coleman-Derr 2018). Additionally, it has been shown that plant growth promoting microorganisms (PGPM) have a greater effect on plant growth during drought compared to well-watered conditions (Rubin, van Groenigen, and Hungate 2017), and it is well established that crops grown in arid desert ecosystems act as "resource islands" for cultivating known PGPM in the surrounding soil (Köberl et al. 2011; Marasco et al. 2012). Recent work has demonstrated that drought has a strong impact on the structure and activity of the root microbiome, and is correlated with a significant enrichment in lineages of monoderm bacteria within the root and rhizosphere that is not observed in the surrounding soil (Naylor et al. 2017; Santos-Medellín et al. 2017; Xu et al. 2018; Fitzpatrick et al. 2018; J. A. Edwards et al. 2018). It should be noted that within these studies, those that investigated single host species (Santos-Medellín et al. 2017; Xu et al. 2018; J. A. Edwards et al. 2018) reported an enrichment of primarily *Actinobacteria*, *Firmicutes* to a lesser degree, and some lineages of *Chloroflexi*. Other studies that looked at multiple host species (Naylor et al. 2017; Fitzpatrick et al. 2018) reported that *Actinobacteria* were the enriched taxa across hosts, and additional studies have

noted that *Actinobacteria* dominate portions of the root microbiome for desert-adapted plants (Marasco et al. 2018). For this reason, the primary focus of this research is on understanding the causes of the *Actinobacterial* enrichment in the endosphere.

It has been shown that applications of *Actinobacteria*, in particular *Streptomyces spp.*, may benefit host fitness under drought (Xu et al. 2018; Yandigeri et al. 2012); many strains are antagonistic toward pathogens (Millard and Taylor 1927; Newitt et al. 2019; Suárez-Moreno et al. 2019), produce beneficial secondary metabolites, and assist in nutrient acquisition (Sathya, Vijayabharathi, and Gopalakrishnan 2017). However, the spatial-temporal dynamics of drought-induced enrichment of *Actinobacteria* remains largely uncharacterized, and it is unclear if this restructuring occurs in all roots—and all parts of each root—within the root system. Water availability is known to vary within the root zone, both at the macro scale (due to the falling water table) and the micro scale (due to the heterogeneous nature of soil composition) (D'odorico and Porporato 2006).Whether the resulting variability in the degree of water stress that is likely to occur across a drought-stressed root system corresponds with differential recruitment of microbes is also currently unknown.

A better understanding of the underlying spatial organization of the observed *Actinobacterial* enrichment may help identify the underlying causes of this phenomenon. At present, it is unknown if the enrichment is driven by local or systemic changes in host physiology or metabolism. If the drought-induced shifts in the root microbiome are limited to roots that directly perceive a lack of water, then localized responses to drought stress could serve as a signal for *Actinobacteria* enrichment. For example, perhaps root tissue death (Y. Liu, Xiong, and Bassham 2009) triggers the proliferation of saprophytic lineages within *Actinobacteria*. Alternatively, if the observed enrichment also occurs in the relatively few roots of drought stressed plants with access to water, this phenomenon may instead be driven by systemic processes, such as above-ground, vasculature-mediated changes in plant metabolism that are translocated throughout all root tissue.

In addition, physiological and functional properties of root tissue differ along the root's longitudinal axis even within the context of a single root (Petricka, Winter, and Benfey 2012). Older root tissue closer to the stem is responsible for root hair and lateral root development, while the youngest tissue at the tip is responsible for active growth, cell division, and is the site of the majority of root exudation (Canarini et al. 2019). Whether the drought-induced enrichment in *Actinobacteria* occurs across the entirety of an individual root's length, or is specific to older or younger tissue types, is currently unknown.

To address these knowledge gaps, we have conducted a series of field and greenhouse-based experiments to allow for spatially-resolved measurements of the compositional shifts within the millet root microbiome that occur in response to drought. Millets are a polyphyletic group of cereal crops that provide a primary source of food and fodder for hundreds of millions of people in the dry regions of Africa and Asia (Patil 2017). They are often grown on marginal lands where irrigation is rain fed and sporadic, and as such are among the crops most exposed to water stress during periods of drought (Kumar et al. 2018). In this study, we worked with five different members of the Paniceae tribe: *Setaria italica* (foxtail millet),

Pennisetum glaucum (pearl millet), *Panicum miliaceum* (proso millet), and *Echinochloa esculenta* (Japanese barnyard millet), which are all millets, and *Sorghum bicolor,* a related cereal crop. We set out to test whether millets, like other cereal crops, are enriched with *Actinobacteria* when drought stressed, and whether this enrichment is correlated with the severity of drought. We also tested whether this pattern is specific to a particular root tissue age, and if enrichment occurs at similar levels from the actively growing root tip to older and more mature root tissue basal to the stem. This would demonstrate whether recently reported drought-induced changes in the plant root microbiome are driven by root specific factors that are independent of the root tissue's developmental stage. Additionally, using a split-pot experimental design, we test whether observed enrichment of *Actinobacteria* is localized to drought-stressed roots, or systematically throughout the root system. Finally, we investigate localized root death as a potential primary driver of the observed bacterial community shifts.

2.2 Results

2.2.1 Bacterial root microbiome is driven by host species and degree of drought

Recent work has shown that drought leads to enrichment of *Actinobacteria* within the root microbiome of a wide variety of angiosperms, including many cereal crops (Naylor et al. 2017; Fitzpatrick et al. 2018). To establish whether drought produced similar enrichment patterns in millets, as well as explore whether such enrichments are correlated with the severity of drought treatment, we conducted a field experiment in which four millet species (see Methods) were subjected to three different watering regimes (control, moderate drought, or severe drought) in a field with acidic silty loam soil (pH 5.2) (Naylor et al. 2017). At the time of sample collection (164 days post-germination), gravimetric soil moisture content was found to be significantly different ($p=1.56E-17$, one-way ANOVA) between all three treatments:16.1% for control (n=12, SD=2.89%), 5.5% for moderate drought (n=12, SD=1.27%), and 3.7% for severe drought (n=12, SD=0.67%). Aboveground phenotypes measured at root collection demonstrate that despite millet's drought tolerance, drought treatment had a significantly negative impact on plant growth (Figures S4-6). Plant height was negatively impacted by drought stress across three millet species (phenotypic data for one species was not collected; Wilcoxon rank-sum test, $p<0.001$), with the greatest impact observed under severe drought stress. Additionally, one variety (*E. esculenta*) displayed a significant reduction in grain ear length (Wilcoxon rank-sum test, $p<0.01$) during severe drought, and median values of ear length decreased with increasing drought severity across all three species. Together these results suggest that drought treatment negatively impacted millet fitness, and that the degree of impact was correlated with drought severity.

To investigate how bacterial communities shifted during increasing levels of drought stress in millet, we profiled the soil, rhizosphere, and root endophyte communities by barcoded amplicon sequencing. We observed that while there is a significant difference between the alpha diversity in bulk soil, rhizosphere, and endosphere samples ($p<0.05$, Tukey's test), there is not a significant difference between the drought treatments within the same sample type (Figure 1A). It is also noteworthy that while the root endosphere communities are less diverse than their corresponding rhizospheres in the control and moderate drought conditions, this is not the case

for severe drought (Figure 1A). Additionally, drought provoked a relative increase in *Actinobacteria* within root endophyte, rhizosphere, and unexpectedly, bulk soil communities (Figure 1B). Moderate drought, which was initiated later in plant development, failed to provoke a strong enrichment in *Actinobacteria* within roots or rhizosphere, a result that is consistent with recent research that demonstrated that drought occurring earlier in development provokes a more substantial shift in *Actinobacteria* (Xu et al. 2018).

To explore how bacterial community composition varied across both host species and treatment, PERMANOVA and ordination analyses were performed on Bray Curtis distances. These analyses revealed significant differences in composition across the dataset are driven by primarily by sample type (F-statistic=17.864, $p<0.001$), with weaker effects contributed by host species (F-statistic=4.952, $p<0.001$), and watering treatment (F-statistic=3.989, $p<0.001$); the strong clustering by sample type is confirmed by Principal Coordinate Analysis (Figure 1C). When considering root endophyte communities alone, the percent of variance attributable to water treatment is 9.6% ($p<0.001$), and the percent of variance attributable to host species is 21.2% ($p<0.001$), and Constrained Analysis of Principal Coordinates reveals clustering by both species and treatment (Figure 1D). Taken together, these results demonstrate that the millet root microbiome responds to drought treatment in a manner similar to other previously reported plant systems, making them suitable systems for the experiments described below.

2.2.2 *Actinobacteria* enrichment pattern occurs along the length of the root

We hypothesized that enrichment of *Actinobacteria* would be observable throughout the root system rather than in specific root zones or types. After profiling the bacterial communities at sub-root system spatial resolution (Figure S1), we found that an enrichment of *Actinobacteria* under drought treatment was observed within single roots and across all three subsections of an individual root, with concomitant decreases in most *Proteobacterial* classes (Figure 2). Additionally, *Actinobacteria* are the predominant indicator taxa of drought within each subsection according to Dufrene-Legendre indicator species analysis (Figure 3) (Dufrêne and Legendre 1997). This demonstrates that *Actinobacterial* enrichment is not unique to the actively growing root tip where most new microbial recruitment to the root endosphere is thought to occur (Shyam L. Kandel, Pierre M. Joubert, and Sharon L. Doty 2017). Notably, however, both *Firmicutes* and *Chloroflexi* appeared more often as indicators of the watered condition, in contrast to what has been observed in several other studies.

Additionally, as part of this experiment, a comparison of intrareplicate and intraplant variation within root sample types was conducted. We hypothesized that due to the stochastic nature of root colonization events and founder effects at smaller physical scales, variation between replicates would be greater in subsections rather than whole root systems. As expected, we observed that as the spatial resolution increases from whole root systems towards individual root subsections, variation between sample replicates increases (Figure S7). Additionally, we observed that root communities of replicates from the same plant are more similar to each other than replicates from different plants (F-statistic=507.4, $p<0.0001$, Figure S8) and replicates from the same root are again more similar compared to replicates from different roots of the same plant (F-statistic=7.453, $p<0.007$, Figure S8). Interestingly, root tips account for greater dissimilarity when comparing subsections of roots both within and between plants, likely

indicating that root tips are sites of stochastic colonization while older middle and basal sections of roots have communities stabilized through selection and competition (Figure S8).

2.2.3 Localized drought causes enrichment of *Actinobacteria*

While the enrichment of *Actinobacteria* bacteria during drought does not appear to depend on the developmental stage of root tissue, it remains unclear whether this enrichment is driven by localized processes at the site of drought, or by systemic responses affecting the entire root system. Using Japanese millet grown in a split-irrigation design (Figure S2), Constrained Analysis of Principal Components (CAP) of amplicon-based bacterial community profiling of the roots and rhizosphere revealed that root endophyte communities from the watered side of the were more similar to the communities of fully watered plants, while the drought-treated side of the split-irrigation plants were more similar to communities found in fully drought-treated plants (Figure 4A, B). Comparisons of the root endophyte community relative abundance patterns between the two sides of split-irrigation plants and between their fully watered and drought treated counterparts demonstrated that there is an increase in the abundance of *Actinobacteria* in drought-treated roots in both full drought and split-drought treatments (Figure 4C). Collectively, these results suggest that *Actinobacterial* enrichment occurs locally at the site of drought induction rather than systemically.

2.2.4 Root death does not drive enrichment of *Actinobacteria*

A subset of *Actinobacteria* lineages can exist as saprophytes (Barka et al. 2016), deriving their carbon from dead and decaying plant material. As localized root tissue death can accompany severe drought stress (Y. Liu, Xiong, and Bassham 2009), we surmised that the observed local enrichment in *Actinobacteria* could be driven by root death. To test this hypothesis, we induced localized root death through mechanical severing and compared levels of *Actinobacteria* recruitment across the root system under drought stress and induced root death treatments (Figure S3). To confirm root death, we used a live-dead stain to test for cell viability. A subset of cells remained viable for three days following root separation, but by nine days cells within separated roots were no longer viable (Figure S9). After community profiling of the root (Figure 5) and rhizosphere (Figure S10) fractions, we observed that in addition to the expected differences in bacterial community composition between drought-treated and watered samples, communities in living or dead tissue showed significant differences. Performing PERMANOVA on the root endophyte samples showed that water treatment explains 23.2% of variance in beta-diversity ($p<0.001$), and tissue death explained 11.6% ($p=0.003$).

Contrary to our hypothesis, an enrichment of *Actinobacteria* was not observed in dead roots compared to living roots under either watering condition (Figure 5). Additionally, performing Dufrene-Legendre indicator species analysis showed that there were no *Actinobacteria* indicators for dead root communities in either watered or drought-stressed tissues. Collectively, these results suggest that their drought-driven shift is unlikely to be attributable to saprophytic activity stimulated through root death. Interestingly, in addition to the expected increase in *Actinobacteria* from watered to drought-stress observed within living roots, a small increase is also observable within the dead roots (Figure 5B). A cell viability assay (Figure S9) demonstrated that a portion of cells within detached roots are still viable after 3 days, suggesting that overall detached roots might be continuing to function metabolically for a period

of time, which could explain the observed slight *Actinobacterial* enrichment if plant metabolism is a primary driver of this phenomenon.

2.2.5 Drought enrichment of *Actinobacteria* is consistent across hosts and drought treatments

We consistently observed enrichment of *Actinobacteria* across multiple experiments including both field and greenhouse studies, multiple millet species, and varying degrees and localizations of drought stress (Figure 6). While *Actinobacteria* as a phylum appears to become generally enriched under drought, other phyla such as the *Proteobacteria* are less consistent in their drought enrichment patterns, with taxa capable of being a significant indicator of both water and drought conditions across different experiments (Figure 6, Table S1). Interestingly, other phyla known to be composed of predominantly monoderm taxa such as the *Chloroflexi* and *Firmicutes* are not enriched under drought and in fact show more significant indicators of watered conditions (Figure 6).

2.3 Discussion

2.3.1 *Actinobacteria*l enrichment under drought occurs irrespective of root tissue age

Our study provides an increased-resolution spatial dissection of the effect of drought stress on the development of the root microbiome and addresses several hypotheses regarding the underlying causes of recently reported increases in *Actinobacteria* that accompany drought stress. Through an exploration of the microbial communities in whole root systems, single roots, and sub-sectioned roots, we found an enrichment of *Actinobacteria* is a common phenomenon along the apical axis of a root (Figure 3). Since the majority of endophytic colonization of the root is thought to occur at the root tip and at positions where lateral roots are emerging (Shyam L. Kandel, Pierre M. Joubert, and Sharon L. Doty 2017), this suggests that the underlying cause of enrichment is not simply increased rates of colonization by *Actinobacteria*, but perhaps also increased proliferation of established *Actinobacterial* endophytes within older root tissue, in comparison to other bacterial phyla. If correct, this implies that any plant-derived molecular signal that contributes to this phenomenon should be present not only within rhizosphere exudates, but also within the endosphere compartment as well. Several proposed molecular mechanisms for the observed *Actinobacterial* enrichment, including shifts in amino acids and carbohydrate biosynthesis and ROS production (Xu and Coleman-Derr 2019), would likely affect both rhizosphere and endosphere compartments.

Other studies have explored how root associated microbial communities change across the apical axis of the root under non-drought conditions (Kawasaki et al. 2016; Baudoin, Benizri, and Guckert 2002; Liljeroth, Burgers, and Van Veen 1991; C. H. Yang and Crowley 2000). In a comparative analysis of microbiome composition between the root tip and root base of nodal roots in *Brachypodium*, Kawasaki et al. identified a relative increase in relative abundance of both *BetaProteobacteria* and *GammaProteobacteria* lineages within the younger, growing root tip as compared to the root base. Interestingly, our data do not display a similar pattern of enrichment for these lineages, which suggests bacterial taxa may have preferential colonization rates at the root tip that differ across hosts or environments.

2.3.2 *Actinobacteria*l enrichment under drought is localized to sites of drought application

While it remains unclear what host mechanisms underlie the cause of the increase in *Actinobacteria* within the root system under drought, our data demonstrate that this enrichment is observed only within roots that are experiencing drought, and not found across the entire root system. For this reason, we propose that host-mediated causes would lie in localized host responses to drought, rather than systemic responses. This would, for instance, potentially exclude shifts in plant metabolites synthesized in the leaves and transported into the root system, that likely result from altered rates of photosynthesis during drought (Pinheiro and Chaves 2011).

Shifts in plant metabolism during drought that are localized to portions of roots subjected to drought have been identified. For example, it was recently shown that in soils with heterogeneous moisture levels, there is an increased accumulation of abscisic acid (ABA), the phytohormone regulator of drought stress response, within roots found in drier regions of soil as compared to those found in regions of higher moisture (Puértolas et al. 2015). The effect of plant-produced ABA on the root microbiome has yet to be determined, though ABA is known to turn on genes for ROS production in the apoplast (Miller et al. 2010; Brito et al. 2019), which could have an impact on the bacterial community (Xu and Coleman-Derr 2019). Perhaps more importantly, ABA acts antagonistically to systemic levels and activity of salicylic acid (SA) (de Torres Zabala et al. 2009) and in turn SA has been shown to influence root microbiome composition (Lebeis et al. 2015; H. Liu et al. 2018). Additionally, it is interesting to note that *Actinobacteria*, such as *Streptomyces*, are known to trigger systemic acquired resistance (SAR), traditionally associated with pathogens (Newitt et al. 2019). Taken together, this suggests that the enrichment of *Actinobacteria* may be driven by a localized hormone mediated response to drought, and that this enrichment itself may drive additional systemic changes in plant immunity.

2.3.3 *Actinobacteria*l enrichment under drought is not driven by root death

Many soil *Actinobacteria* function as saprophytes, consuming dead organic material (Barka et al. 2016). We had hypothesized that *Actinobacteria* may perceive root death within the drought-stressed root system and that this triggers their increased activity and abundance. However, we demonstrate that microbial communities of severed roots had fewer *Actinobacteria* than intact roots under both watered and drought treatments; in fact, *Actinobacteria* are the predominant indicators of living roots tissue. It is possible saprophytic colonization and activity does contribute to long term *Actinobacterial* increases under drought, and that such shifts take longer to develop than the time frame used in this study.

While historically categorized as free-living saprophytes, recent work on the root microbiome suggests that many *Actinobacteria* may have a less well understood phase of development or lifestyle associated with the endosphere, which leads to alternate functions and potentially even changes in cellular morphology (van der Meij et al. 2018; Ramijan et al. 2018). It is known that some bacteria occupy different niches (i.e., play different functional roles) depending on the presence of certain environmental triggers, such as carbon sources (Duffy and Défago 1999; Sánchez et al. 2010). Indeed, some *Actinobacterial* lineages long considered saprophytic have been shown to, under certain environmental conditions, enhance plant growth through competition with plant pathogens (Millard and Taylor 1927; Newitt et al. 2019). Since it

is unknown what triggers the switch to a saprophytic lifestyle, and *Actinobacteria* are abundant in both living and dead roots, we could hypothesize that the bacteria are attracted to inert components of plant cell walls that are present under both conditions, and the endophytes do not express saprophytic functions within the living root environment.

2.3.4 Variation in enriched genera within *Actinobacteria*

Drought-induced enrichment of *Actinobacteria* has been observed in this study across multiple experiments with different host plants, which supports a growing body of evidence that this is a widespread pattern during drought (Xu et al. 2018; Naylor et al. 2017; Fitzpatrick et al. 2018; Santos-Medellín et al. 2017; J. A. Edwards et al. 2018). Additionally, our work supports previous studies that show differences in enrichment at finer taxonomic resolution (Naylor et al. 2017; Fitzpatrick et al. 2018). That is, though *Actinobacteria* show consistent enrichment as a phylum, the families and genera that are enriched may vary between host plants or experiments (Figure 6). *Streptomyces* is perhaps the most notable *Actinobacteria* genus that has been described to have plant-growth promoting abilities, particularly during abiotic or pathogen stress (Xu et al. 2018; Yandigeri et al. 2012; Qin et al. 2015; Singh et al. 2016; Newitt et al. 2019). While *Streptomyces* are known to produce spores, previous studies have ruled out spore-production as the sole explanation for *Actinobacterial* enrichment under drought as there are other enriched *Actinobacterial* genera that do not contain the genetic prerequisites for spore formation (Naylor et al. 2017). Additional dissection of the host and microbial molecular response to drought stress using a combination of genetic and omic tools may help to narrow down the underlying cause of this phenomenon.

2.4 Materials and Methods

2.4.1 Drought gradient and multi-species field design

Four species of millet—all members of the *Paniceae* tribe—were planted on May 19th, 2015 at the University of California at Berkeley's Gill Tract research field in Albany, California (37°53'12.3"N 122°18'00.3"W): *Setaria italica* (foxtail millet), *Pennisetum glaucum* (pearl millet), *Panicum miliaceum* (proso millet), and *Echinochloa esculenta* (Japanese millet). Seeds were planted directly in the field with 8-10 seeds per hill and hills 25-30 cm apart. The four species were subjected to three different watering regimes: control (watered on the day of planting then weekly until maturity), moderate drought (watered on the day of planting, weekly for the next five weeks, and water then withheld until maturity), and severe drought (watered on the day of planting, once the following week, then withheld until maturity). Watering treatments were applied for 6 h using drip irrigation tape with 1.89 L/h rate flow emitters. Tissue and soil samples were harvested 24 weeks post-germination, after each species had reached maturity. Root systems for each species are structurally similar; they all are fibrous and lack a tap root, typical of monocotyledons. Bulk soil samples were taken 30 cm from the base of the plant at the same time point; root/rhizosphere were collected as detailed in (Simmons et al. 2018) and stored in phosphate buffer at -80°C until further processing.

2.4.2 Sub-sectioned root field experimental design

S. bicolor was chosen for this experiment due to its larger root structure, which allowed for increased precision during root system dissection. *S. bicolor* seeds were planted on June 21, 2017

at the USDA Gill Tract research field in Albany, California under a sheet of plastic mulch to reduce weed growth. Plants were watered weekly for the first three weeks after planting. For each application, water was administered for 6 h using drip irrigation tape with 1.89 L/h rate flow emitters. Samples were taken for whole root systems and three single roots after 1 week. After 2, 3, 9, and 11 weeks, we collected whole root systems and six single roots, three of which were further partitioned into 3 approximately equal length subsections. At each time point, a single bulk soil sample was collected for each plant, approximately 30 cm from the base of the plant. When collecting root samples, the single roots were collected first, and the remaining roots were pooled and considered to be the whole root system (Figure S1). The single roots were selected from the system by: presence of root tip and minimal lateral root growth. After sample collection, roots were placed into sterile conical tubes with phosphate buffer and stored at -20°C until further processing.

2.4.3 Split-pot experimental design

Fifteen *E. esculenta* seeds were planted in sterile pots filled with sifted field soil and grown for 2 weeks before transferring 12 plants to a split-pot design (Figure S2). The split-pot design consisted of two 1-L square sterile pots connected together with adhesive and filled with field soil pre-sifted through a 1 cm sieve; transplanted seedling roots were partitioned such that half of the root system was located on each side of the split-pot system. After a one week acclimation period, three different watering regimes were initiated: full water (W; water was applied on both sides), full drought (D; drought was applied on both sides), and half-water/half-drought (W/D; water was applied only on one side) with four plants per treatment. A plastic sheath was applied to the outside of the pot on the drought side of the W/D plants to prevent water from moving up through the base of the pot from the water reservoir below. Plants were grown for an additional 10 days before collecting bulk soil from both sides of the pot; root/rhizosphere samples were collected as described above and stored in phosphate buffer at -20°C until further processing.

2.4.4 Live-dead root community profiling design

Five *E. esculenta* seeds were planted per pot in 0.25L sterilized pots (13) in a greenhouse. After one week, the pots were thinned to one plant each, and one week later the plants were transplanted to sterile 4 L pots filled with sifted (1 cm sieve) field soil. They were grown for an additional week before initiating drought stress on half of the plants (28 days post-germination). One day after the start of drought treatment, a subsection of roots was severed from the rest of the plant by connecting a razor blade to the end of a wooden stake and pushing it at a 45˚ angle through the root zone, starting at the base of the plant (Figure S3). The blade was then removed, and the wooden stake replaced within the soil to identify the location of separated tissue. After 10 days of drought, root and rhizosphere samples were collected from both living and dead roots and placed into conical tubes with phosphate buffer. Samples were stored at -20°C until further processing. Additional severed and live root samples were collected from replicate plants to perform cell viability assays on the roots. These assays were performed on roots collected on the day of root detachment, three days later, and on the day samples were collected for community profiling (nine days post-detachment). To assay cell death, we used the Plant Cell Viability Assay kit (Sigma-Aldrich, Darmstadt, Germany) according to the manufacturer's instructions.

2.4.5 Root/rhizosphere processing and DNA extraction

The methods used here are modifications of what is described in Appendix A (Simmons et al. 2018). Roots frozen in phosphate buffer solution were thawed at 4°C and washed by sonication in a Bioruptor Plus ultrasonicator (Diagenode, Denville, NJ, United States) at 4°C for 10 minutes. Roots were removed from vials and rinsed twice with autoclaved water. For each plant in the subsectioning experiment, three of the clean individual roots were then cut into three sections of equal length. Roots not being processed immediately were placed in fresh sterile phosphate buffer and frozen at -80°C. Rhizosphere soil samples from the sonicated vials were centrifuged (10 min at 4 °C, 4,000 x g), and DNA was extracted by processing approximately 250mg of each sample with MoBio's PowerSoil kit (prior to Qiagen purchasing MoBio). DNA was extracted from root samples by grinding to a powder with liquid nitrogen, mixing 600-700mg powder with CTAB buffer, and washing with phenol chloroform-isoamyl alcohol. For individual and sectioned roots in the subsectioning experiment, DNA was extracted using approximately 50mg of tissue in MoBio's PowerPlant kit. Bulk soil DNA was extracted with MoBio's PowerSoil kit.

2.4.6 16S Amplification and Sequencing

All samples were amplified in triplicate using barcoded universal primers (180 s at 98 °C, 30 cycles of: 98 °C for 45 s, 78 °C for 10 s, 55 °C for 60 s, and 72 °C for 90 s, then 600 s at 72 °C followed by a 4 °C hold) for the v3-v4 region (341 F, 5'-CCTACGGGNBGCASCAG-3' and 785 R, 5'-GACTACNVGGGTATCTAATCC-3') of the 16S rRNA gene according to (Simmons et al. 2018). Additionally, PNAs matching chloroplast and mitochondrial 16S sequences were spiked into PCRs (2.28 μM final concentration) to prevent amplification of these unwanted reads. Replicate PCR products were pooled and quantified using Qubit HS assay; 100ng from each sample was pooled together and cleaned using AMPureXP magnetic beads before a final quantification and dilution to 10nM for sequencing at the UC Berkeley Vincent Coates Sequence Facility via Illumina MiSeq (v3 chemistry, 300 bp paired-end sequencing). Reads were demultiplexed in QIIME2 (Bolyen et al. 2018) and then passed to DADA2 (Callahan et al. 2016a) where sequences were trimmed to ensure minimum median Phred Q-scores of 30 or greater at any given base pair position prior to denoising and Amplicon Sequence Variant (ASV) inference; 500,000 reads were used to train error-rate models, but otherwise all other pipeline default settings were used. A taxonomy classifier was trained to the V3-V4 region of sequences from the August 2013 version of GreenGenes 16S rRNA gene database via Naive Bayesian methods in QIIME2 and used to assign taxonomic associations to ASVs. All subsequent statistical analyses were completed in R; scripts and datasets can be found at https://github.com/colemanderr-lab/Simmons-2020. The phylogenetic tree of indicator species was generated using the online tool: Interactive Tree Of Life (iTOL) v5 (Letunic and Bork 2019). All raw reads are deposited in the NCBI Short Read Archive at accession PRJNA607579.

Chapter 2 Main Figures

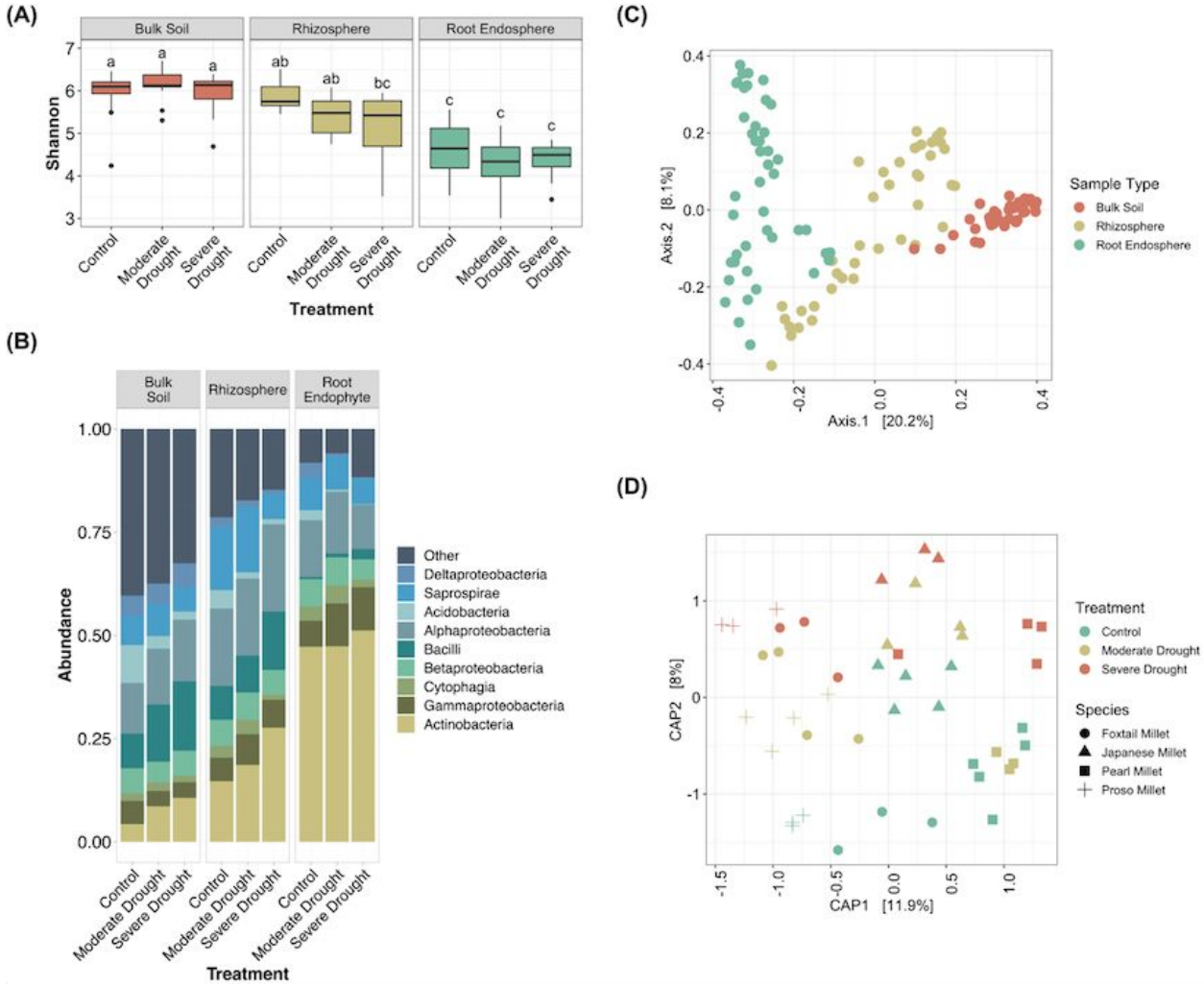

Figure 1.
Millet root microbiome profiles vary by severity of drought. A) Box-and-whiskers plot of Shannon's diversity of bulk soil, rhizosphere, and root endophyte communities subjected to a gradient of water conditions (control, moderate drought, and severe drought). Letters indicate significantly different groups ($p<0.05$, Tukey's test) From left to right sample size: n=13, 13, 13, 12, 12, 15, 16, 15, 13. B) Relative abundance of the top 9 most abundant bacterial classes in each compartment of the root microbiome. C) Ordination plot (PCoA) of all samples based on Bray-Curtis dissimilarity, colored by the source material. D) Constrained ordination plot (CAP) of root endophyte samples based on Bray-Curtis dissimilarity. Colors indicate treatment type and shape indicates host species.

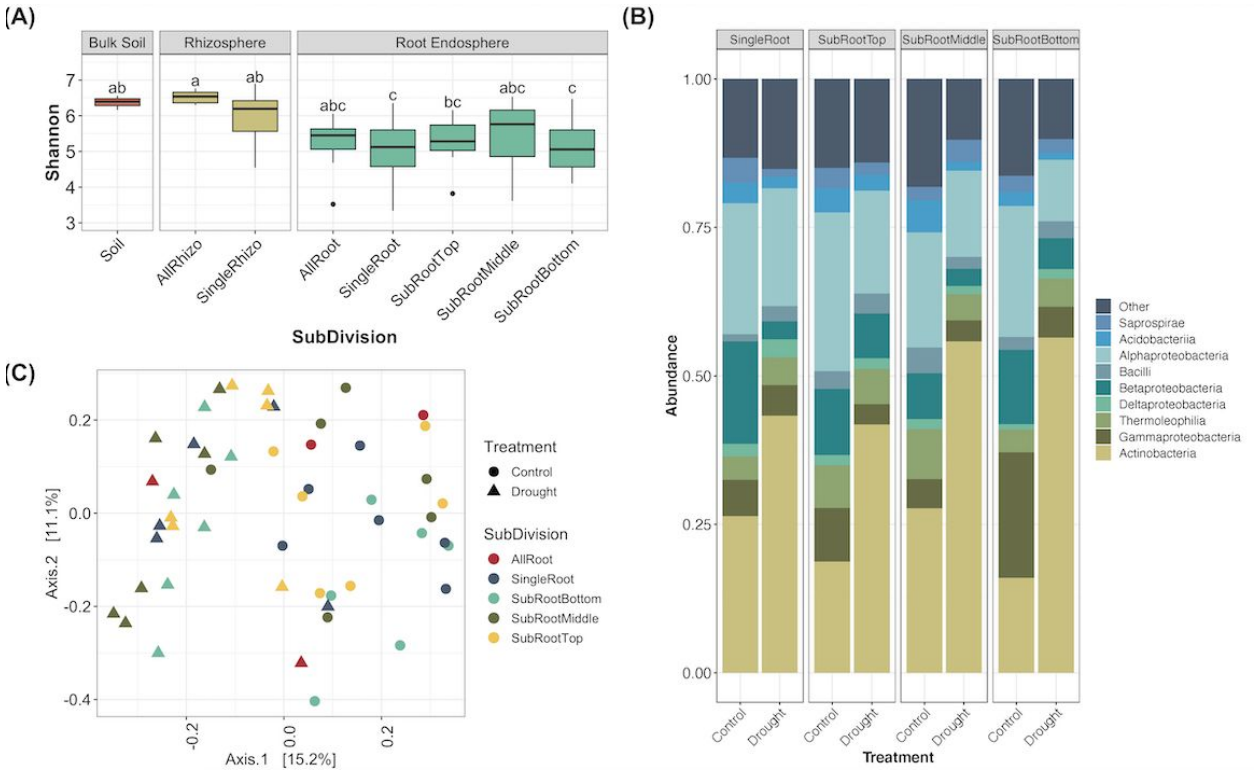

Figure 2.
Variation in sorghum root microbiome communities by root age. A) Box-and-whiskers plot of Shannon's diversity of bulk soil, rhizosphere, and root endophyte communities separated by root age. SubDivision (x-axis) indicates whether samples came from whole root systems (AllRoot; the bulk of roots remaining after six individual roots were collected), individual roots (SingleRoot), or subsectioned roots (SubRootTop is closest to the plant and SubRootBottom is the root tip). Letters indicate significantly different groups ($p<0.05$, Tukey's test) . B) Relative abundance of the top 9 most abundant bacterial classes in the root endosphere separated by water treatment. C) PCoA plot of root samples based on Bray-Curtis dissimilarity, colored by root age where open circles are well-watered control roots and triangles are drought-stressed roots.

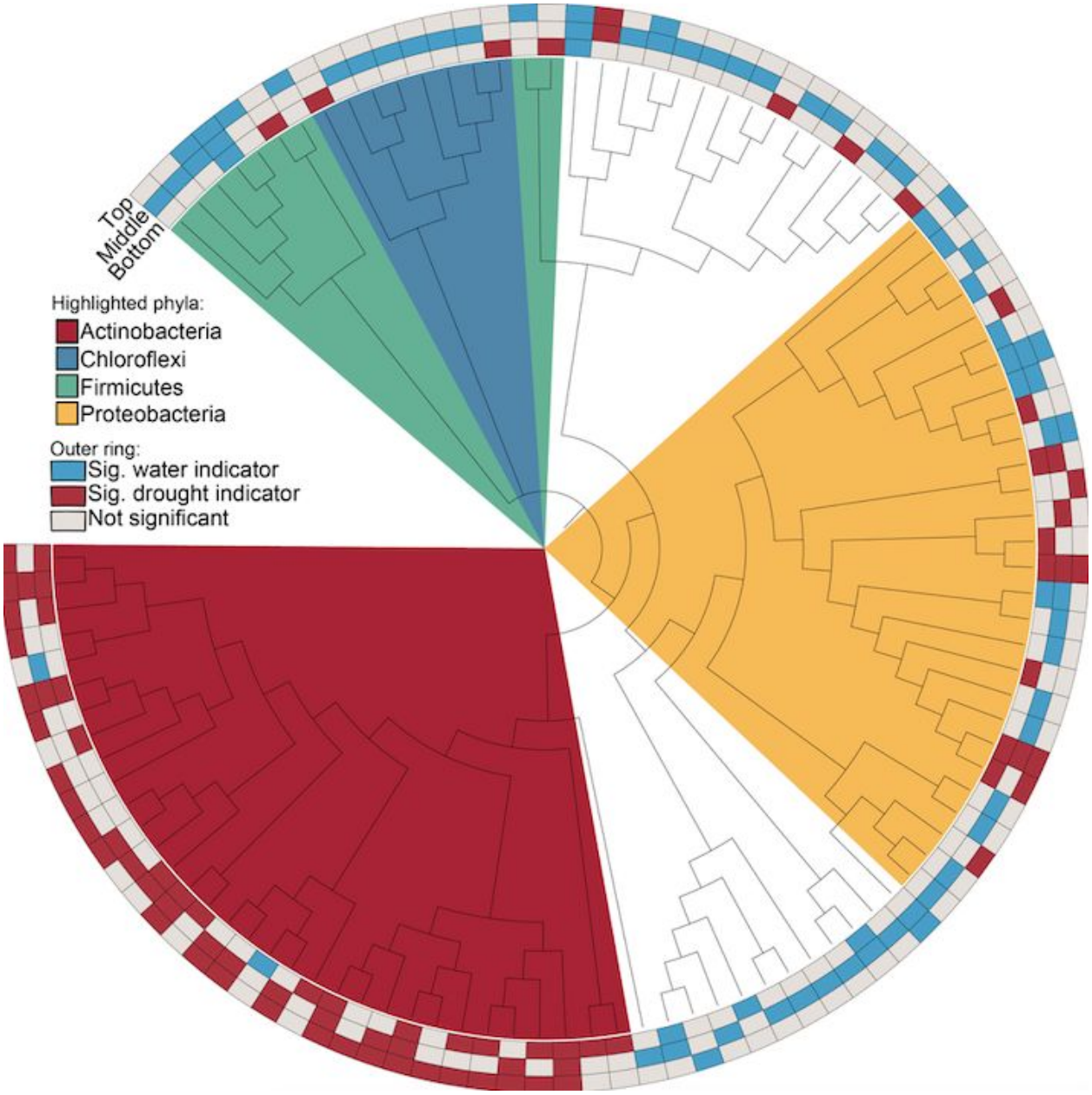

Figure 3. Indicators of drought and watered conditions by root subsection (root tip, middle, or base). Nodes represent genera where taxonomic information was available to group ASVs by this rank. Red and blue boxes indicate nodes that are significant indicators of drought and water conditions after Dufrene-Legendre analysis, respectively ($p < 0.05$, indcls > 0.5). Nodes are highlighted by phyla (*Actinobacteria* (red), *Chloroflexi* (blue), *Firmicutes* (green), *Proteobacteria* (yellow)).

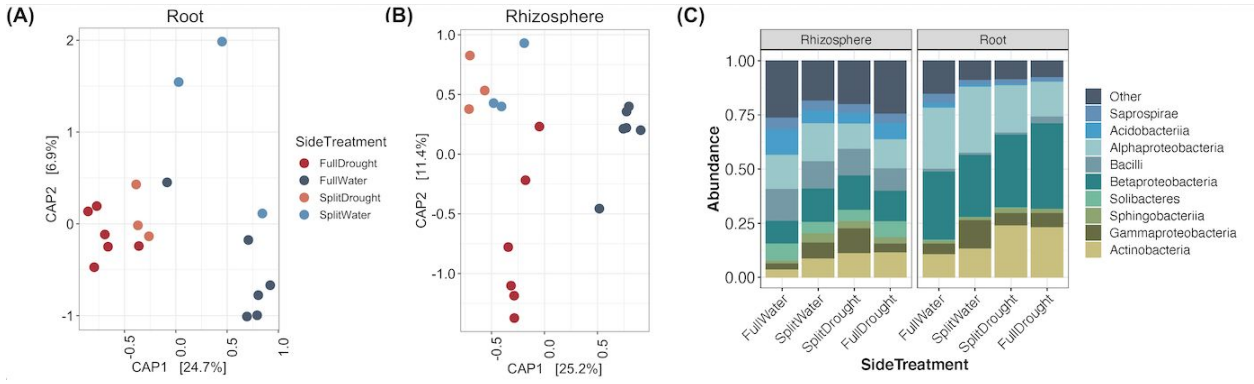

Figure 4.
Effect of a split-pot watering system on the root microbiome. A) CAP plot of root endosphere samples colored by water treatment in Japanese millet. B) CAP plot of rhizosphere samples colored by water treatment in Japanese millet. C) Relative abundance of the top 9 most abundant bacterial classes in either the rhizosphere or endosphere of the different watering treatments of Japanese millet.

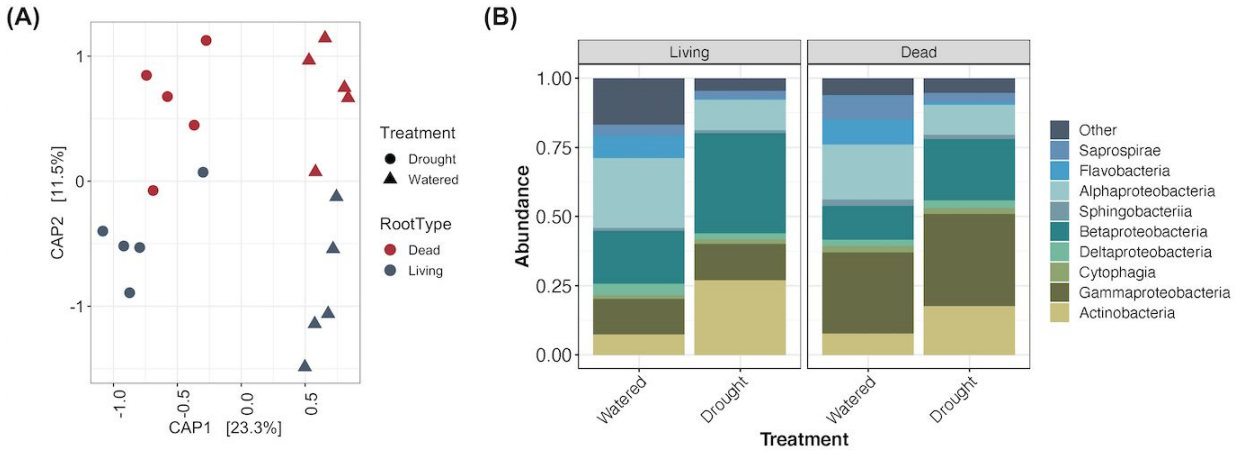

Figure 5.
Impact of root death on Japanese millet root endophyte communities. A) CAP plot of root endosphere samples colored by root type and shaped by water treatment. B) Relative abundance of the top 9 most abundant bacterial classes in the root endosphere separated by root type and water treatment.

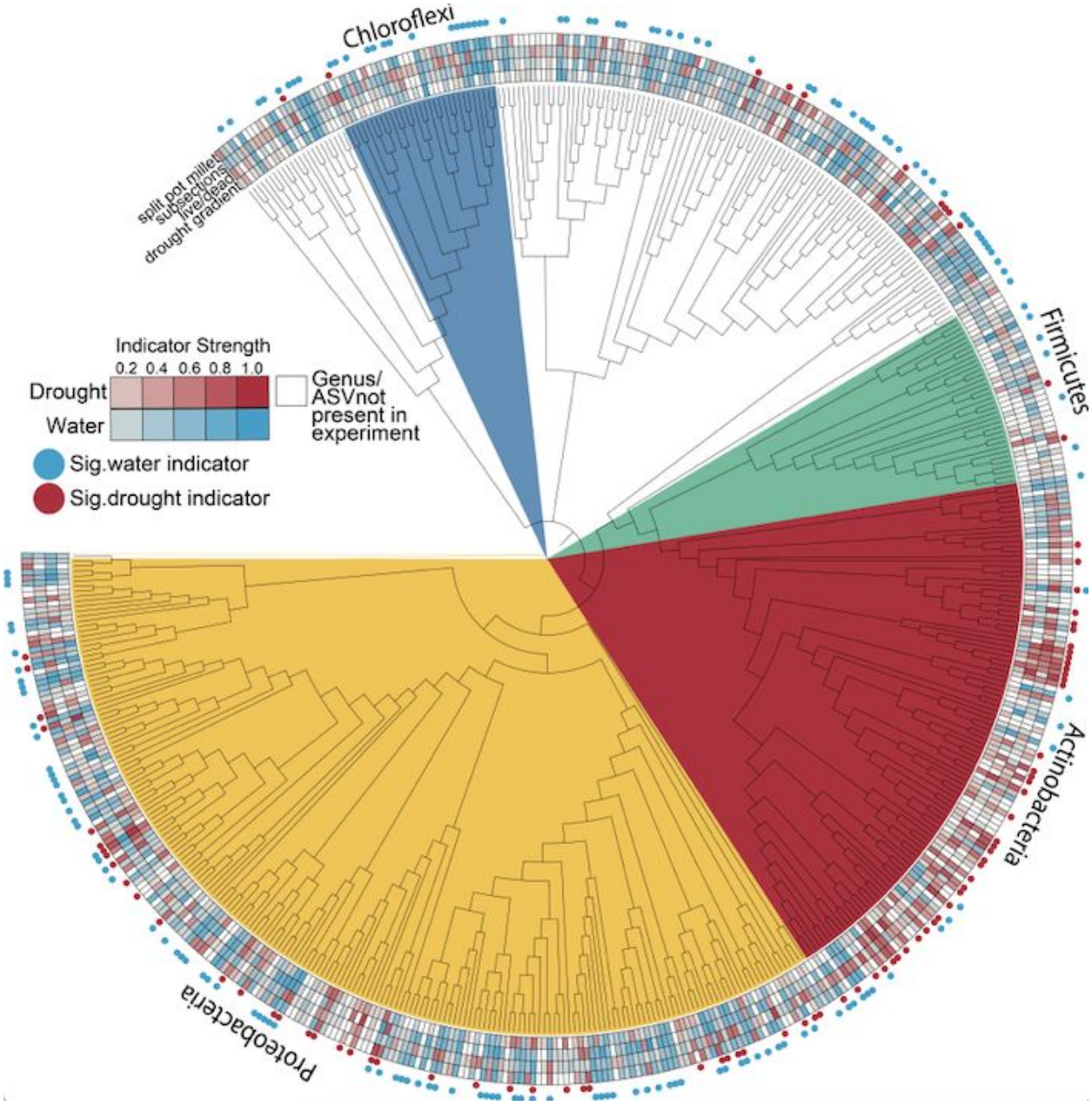

Figure 6.
Root endophyte indicator values for drought and water conditions across all experiments. Nodes represent genera where sufficient taxonomic information was available to group ASVs by this rank; ASVs without genus-level annotations were included but ungrouped. Heatmaps connote indicator strength for drought and watered conditions (red and blue, respectively; white indicates taxa not present in a given experiment) and filled circles connote taxa that were a significant indicator of drought (red) or watered (blue) conditions in at least one experiment ($p<0.05$, indcls > 0.5).

Chapter 2 Supplemental Figures

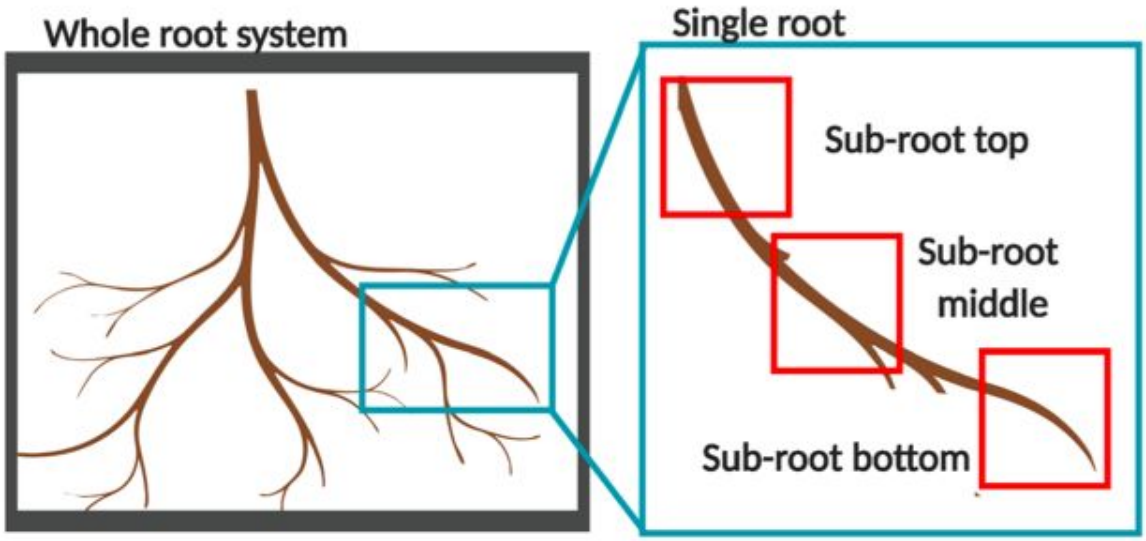

Figure S1.
Diagram showing sampling method of roots and root subsections from sorghum roots. After the plant was harvested, six individual roots were separated (three for Single Root samples, and three to be sub-sectioned), and the remaining roots were pooled together. Created with BioRender.

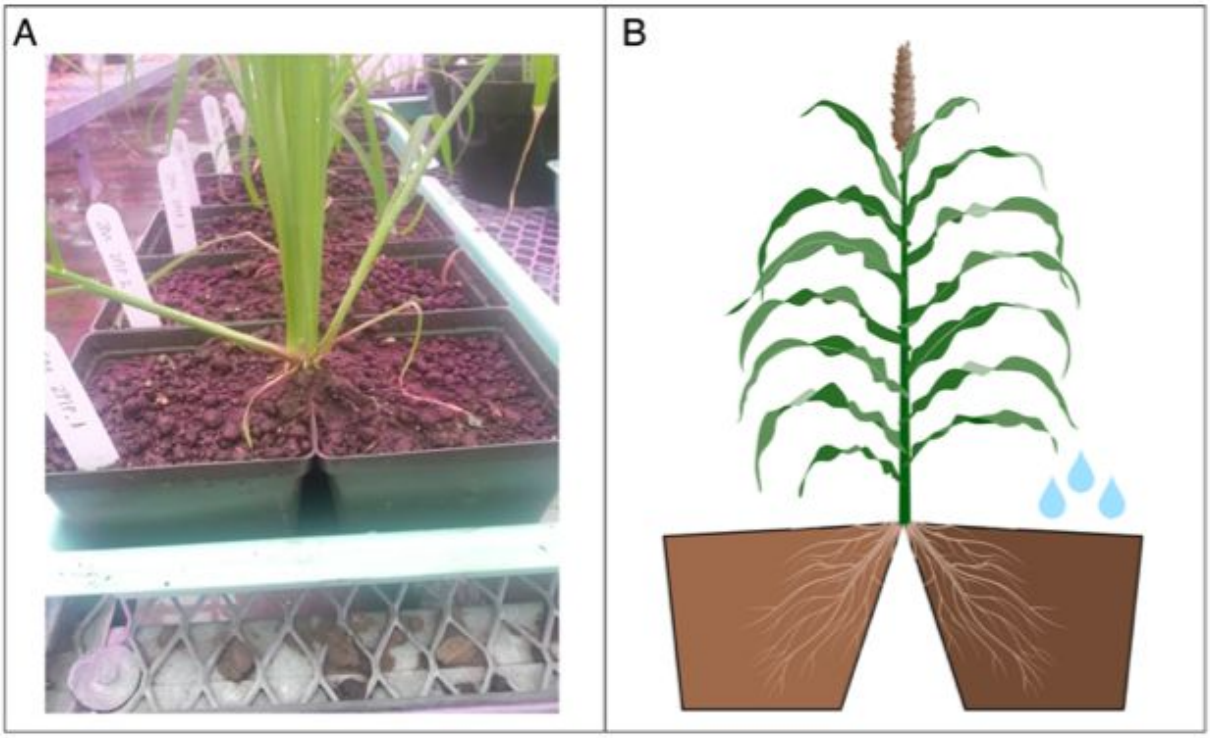

Figure S2.
Image taken from greenhouse set-up of Japanese millet growing in a split-pot system (A) and a diagram showing the watering regime for the split-pot treatment (B). Created with BioRender.

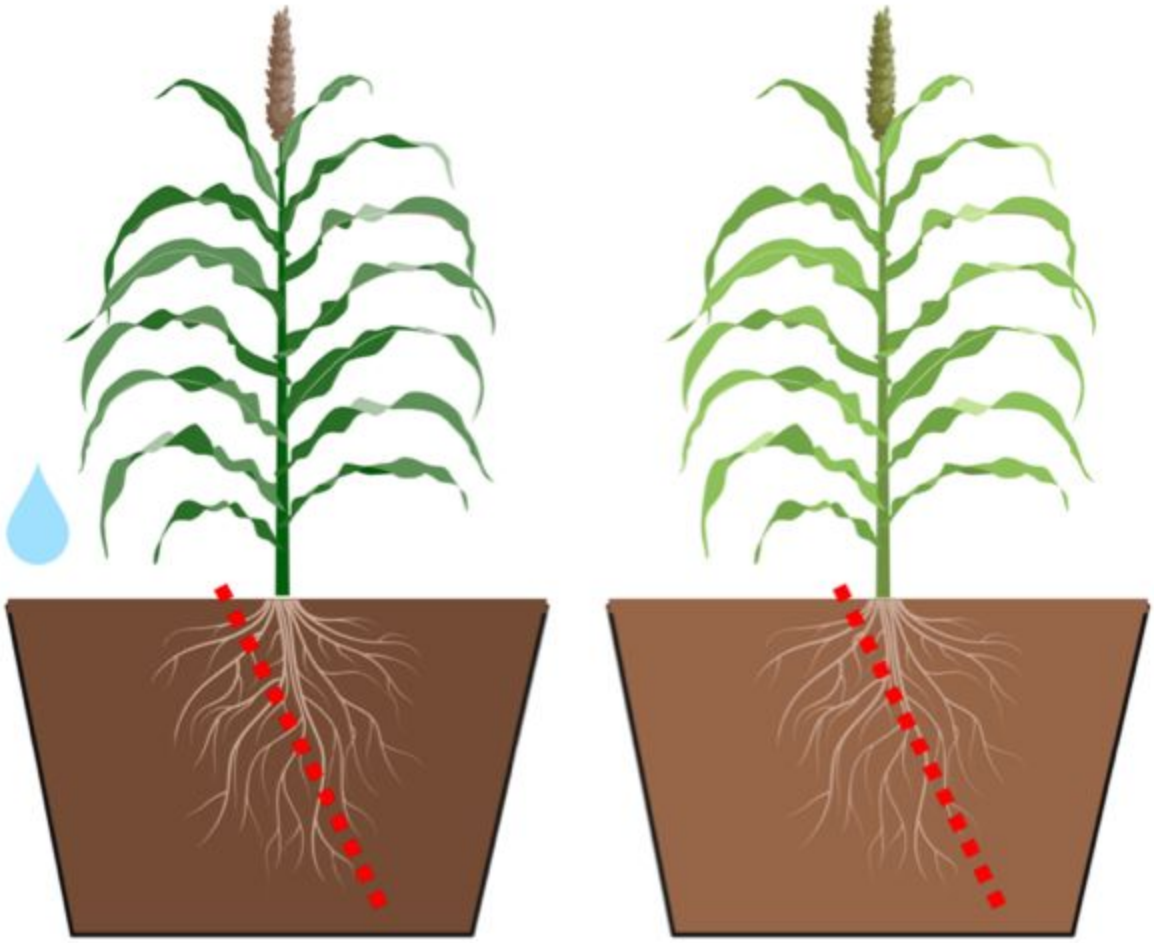

Figure S3.
Diagram showing set-up for live-dead root experiment. Blade was pushed through the roots approximately along the dotted red line, and half the plants were subjected to drought stress. Created with BioRender.

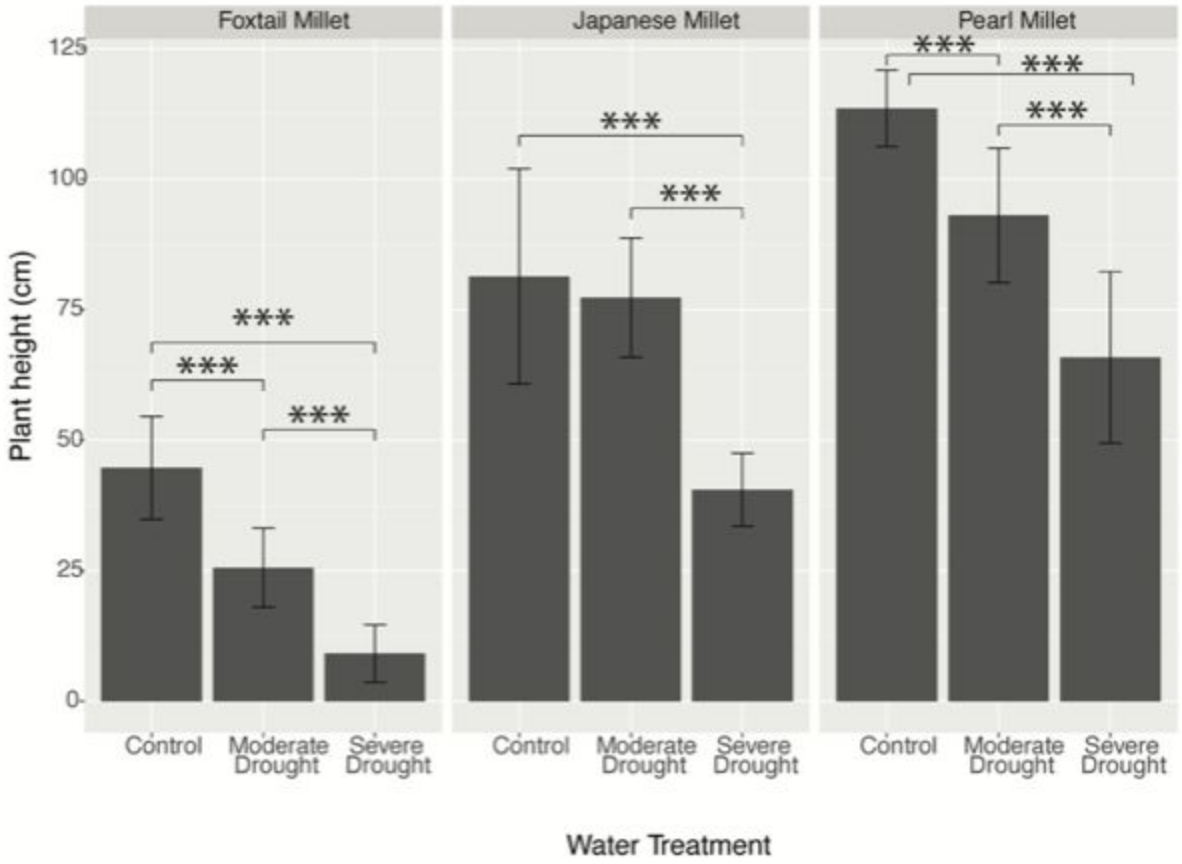

Figure S4.
Phenotypic data from multi-species millet field experiment. Plant height in centimeters (n=25). Vertical lines show standard deviation, and asterisks indicate statistical significance (*=$p<0.05$, **=$p<0.01$, ***=$p<0.0001$) according to the Wilcoxon rank sum test. Data is not available for proso millet due to destruction by native fauna.

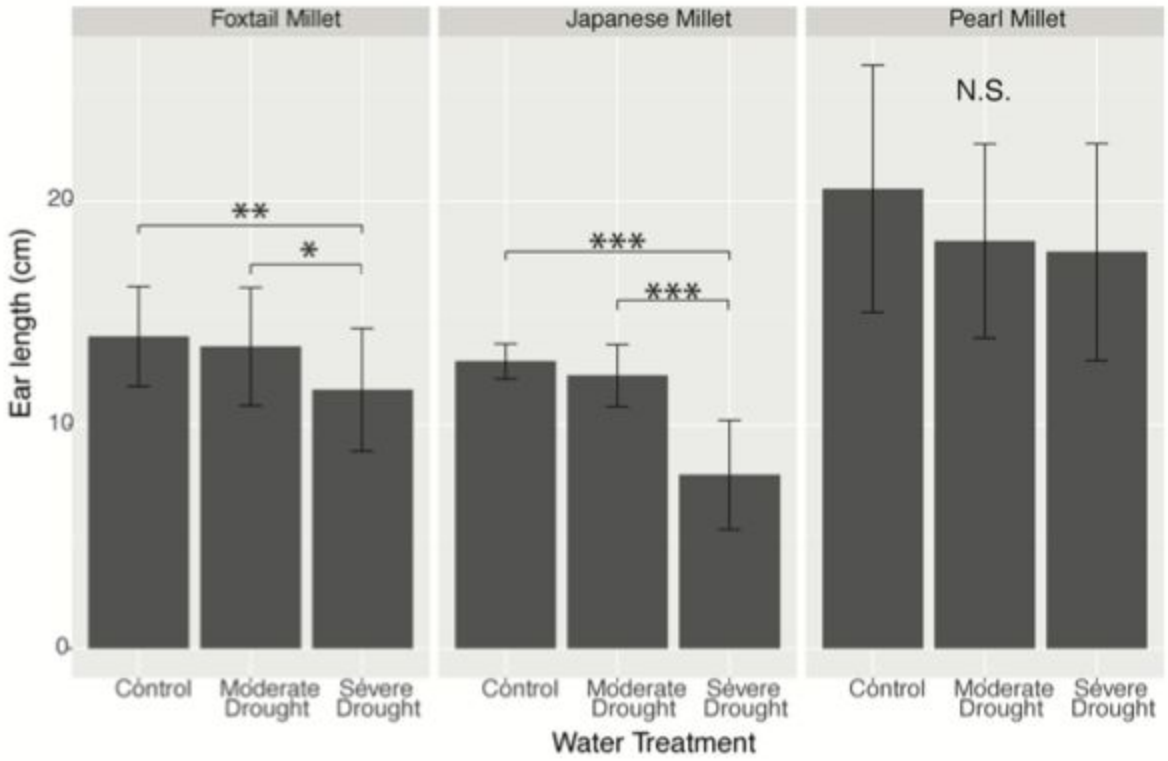

Figure S5.
Phenotypic data from multi-species millet field experiment. Ear length in centimeters (n=25). Vertical lines show standard deviation, and asterisks indicate statistical significance (*=$p<0.05$, **=$p<0.01$, ***=$p<0.0001$, N.S.=no significance) according to the Wilcoxon rank sum test. Data is not available for proso millet due to destruction by native fauna.

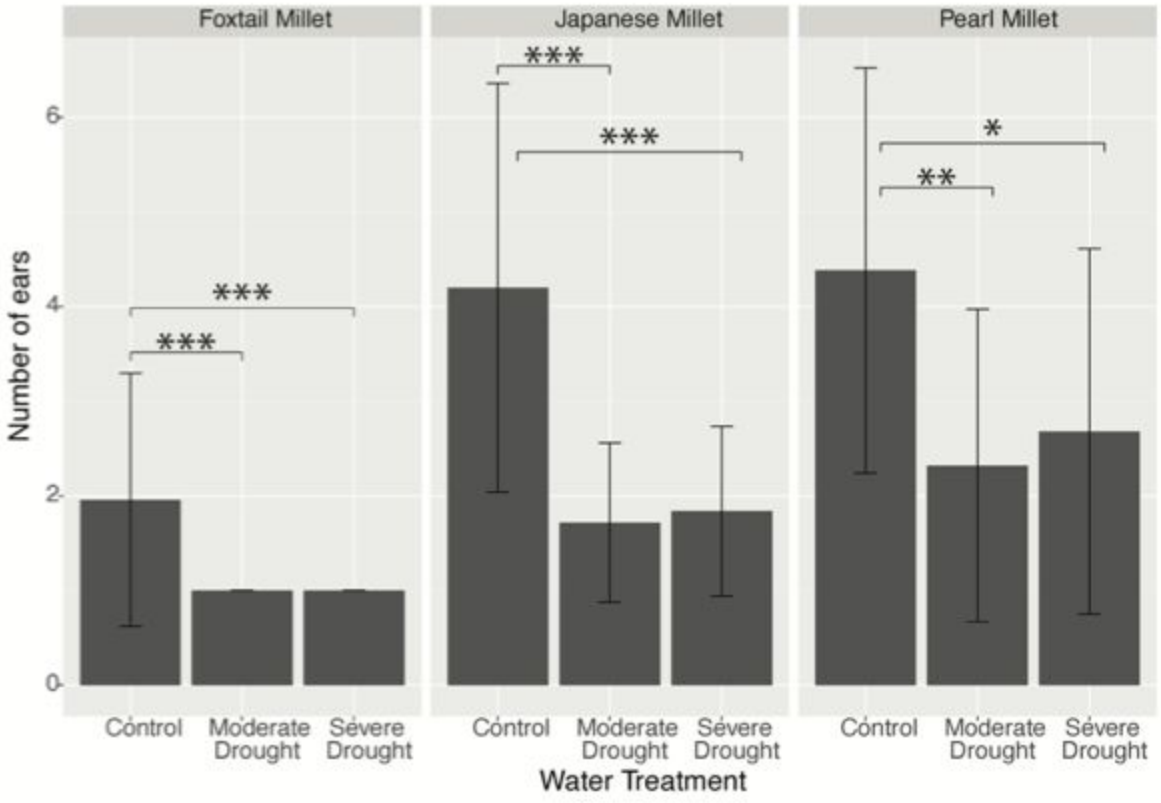

Figure S6.
Phenotypic data from multi-species millet field experiment. Number of ears per plant (n=25 except Pearl Millet Control, n=23). Vertical lines show standard deviation, and asterisks indicate statistical significance (*=$p<0.05$, **=$p<0.01$, ***=$p<0.0001$) according to the Wilcoxon rank sum test. Data is not available for proso millet due to destruction by native fauna.

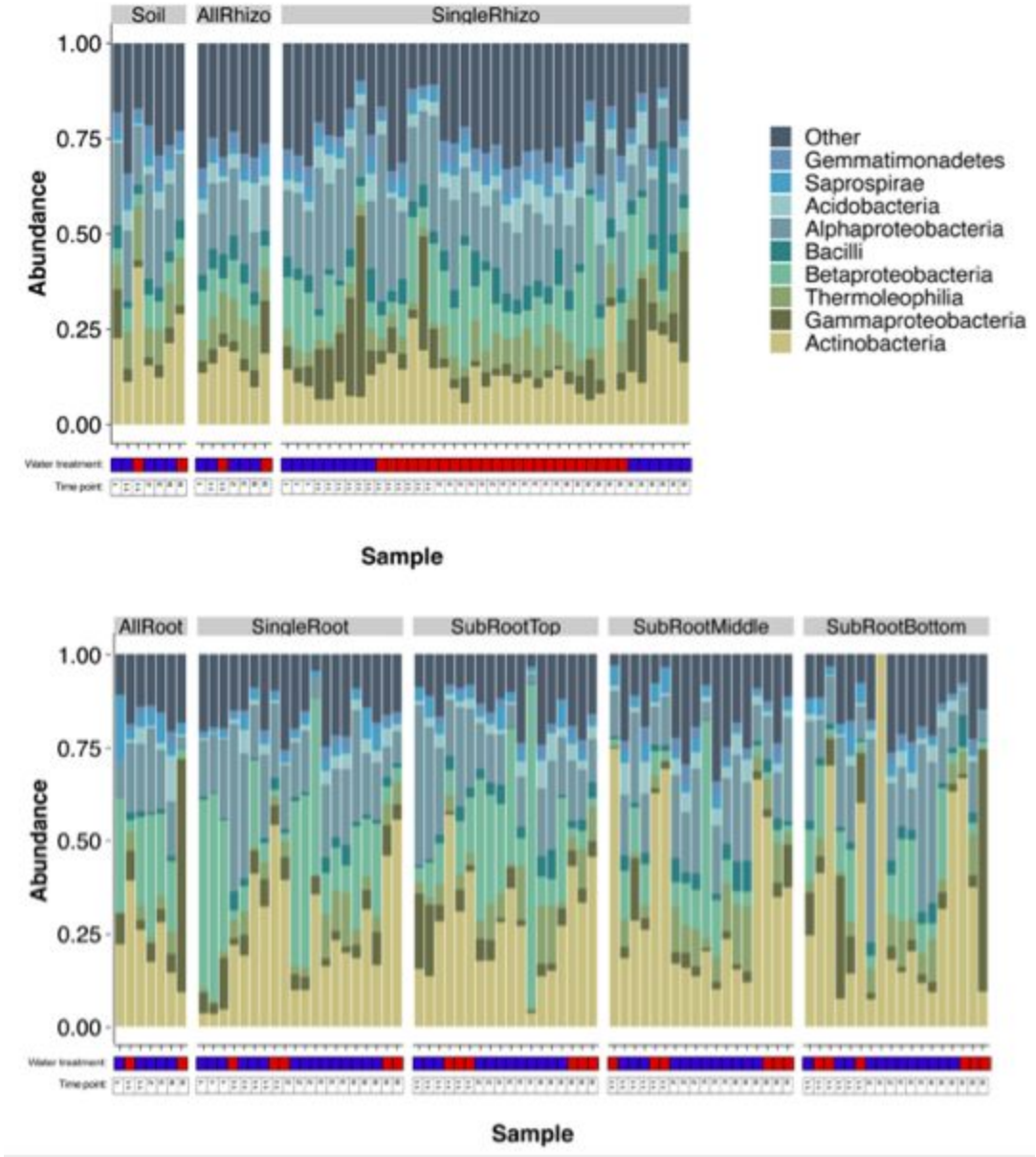

Figure S7.
Relative abundance of the top 9 most abundant bacterial classes within all samples in the subsection experiment. Each column represents a single sample. Colored blocks below columns indicate whether the sample was under well-watered (blue) or drought (red) conditions. Numbers under colored blocks represent the time point the sample was collected (in weeks).

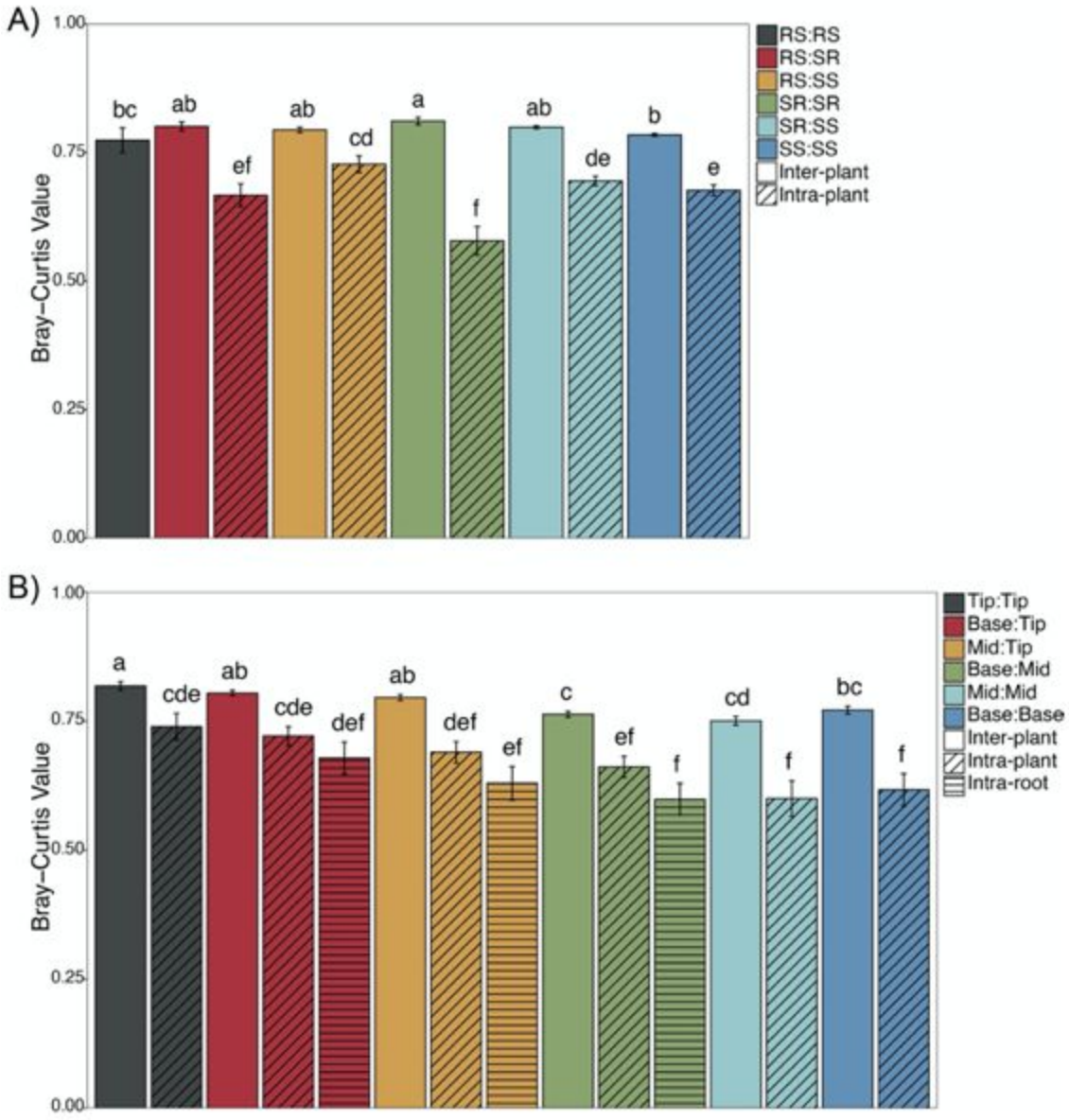

Figure S8.
Bray-Curtis dissimilarities between different sample types: RS – whole root system, SR – single roots, SS – subsection (A); in (B) all samples are subsections further classified as root tips, root middles, and root bases. In both (A) and (B), no matter the sample type, comparisons between samples of the same plant are more similar compared to samples from different plants. (B) additionally shows that samples from the same root are more similar compared to samples from different roots from either the same or different plants.

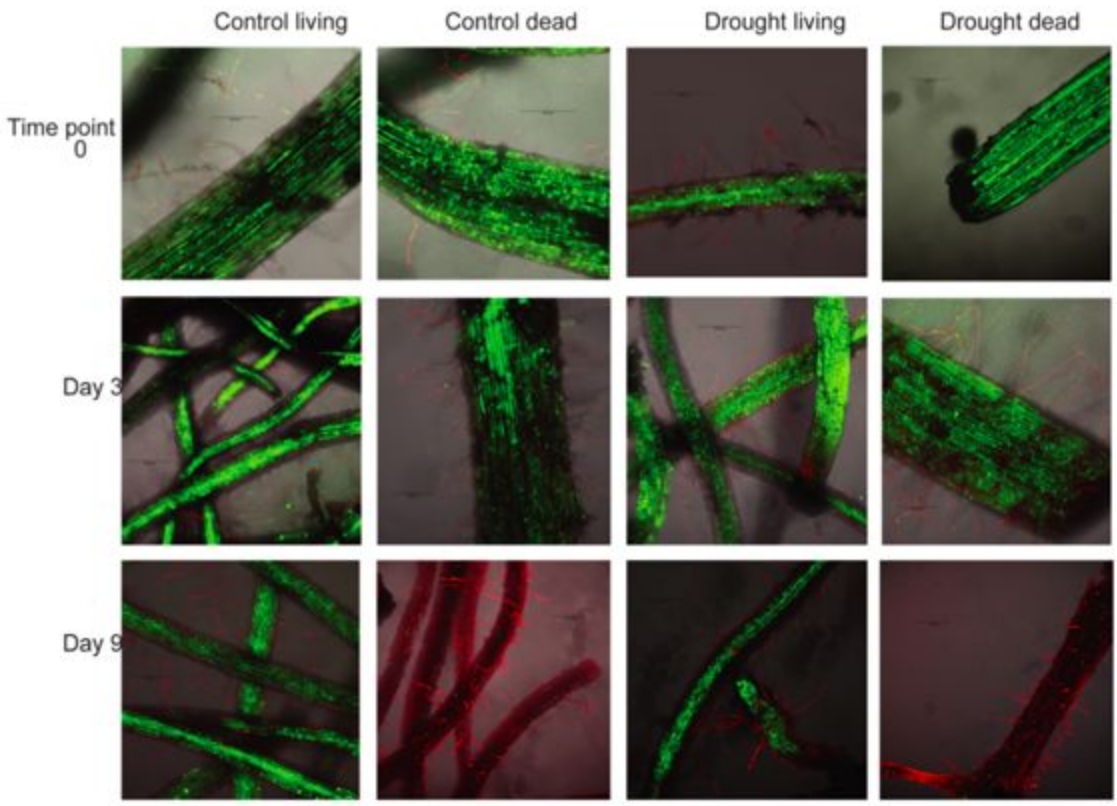

Figure S9.
Fluorescence microscopy of roots in live-dead experiment using Sigma-Aldrich's Plant Cell Viability Assay kit. Green indicates intact cell membranes and red indicates ruptured cell membranes. Assay was performed on living or detached roots in either drought or control water conditions on the day of detachment (time point 0), and 3 and 9 days post-detachment.

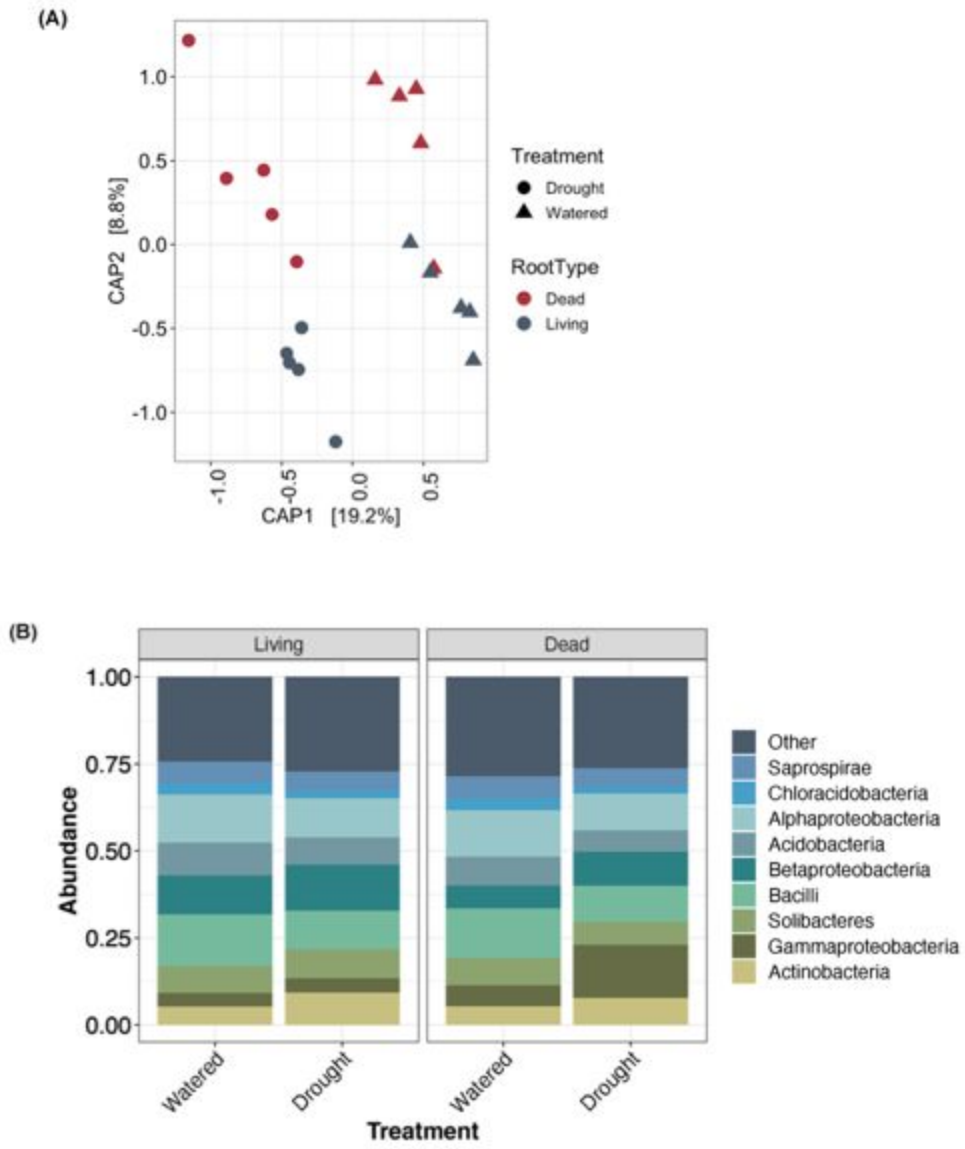

Figure S10.
Impact of root death on Japanese millet rhizosphere communities. A) CAP plot of root endosphere samples colored by root type and shaped by water treatment. B) Relative abundance of the top 9 most abundant bacterial classes in the root endosphere separated by water treatment.

Table S1.
Numbers of indicators for drought and water conditions across all experiments tabulated phylum. For this analysis ASVs were grouped by genera where taxonomic information was available at this level; nodes, then, represent both merged genera and ASVs unannotated at the genus level. These are the top 10 phyla by total number of nodes.

	# Drought Indicators	# Water Indicators	Drought/ Water	Total nodes representing phyla
Acidobacteria	1	10	0.1	23
Actinobacteria	40	14	2.86	110
Bacteroidetes	7	18	0.39	41
Chloroflexi	1	12	0.08	27
Cyanobacteria	0	2	0	5
Firmicutes	2	6	0.33	35
Gemmatimonadetes	0	6	0	8
Proteobacteria	38	74	0.51	201
TM7	1	1	1	8
Verrucomicrobia	0	9	0	20

Chapter 3: Developing a tool - an *Actinobacteria* rich strain library

3.1 Introduction

In recent years, research in environmental microbiology has been focussed on *in situ* studies that use -omics technologies (metagenomics, transcriptomics, proteomics, etc.). While we've learned a great deal from these studies, this knowledge can now be used to dive deeper into the basic microbiology. With multiomics, we have clues as to what these microbes are consuming (Garza et al. 2018) or the roles they play in their environment (X. Gao et al. 2018). However, we are less confident in determining how two strains are interacting with each other or which community members produce particular metabolites. For years, microbiologists have been plagued with "the great plate count anomaly" (Staley and Konopka 1985); even before the advent of metagenomics, scientists could see microorganisms under the microscope that they were not able to cultivate. When the concept of amplicon sequencing of a universal gene first introduced in 1985, existence of novel taxa, never before cultured, were confirmed from environmental DNA (Pace et al. 1986). These studies provided invaluable information about microbes that have yet to be cultivated. However, there are still limitations to what we can learn using these -omics techniques, and validating these discoveries still requires growing isolates in the lab.

There are several reasons why scientists might not be able to cultivate a bacterium in a laboratory setting. In some cases, organisms grow so slowly that they are outcompeted by faster growing organisms. If samples are diluted to capture these organisms, plates may still be discarded before colonies become visible, and in the case of rare community members, it may require hundreds or thousands of plates before they are statistically likely to be found. Another problem arises when nutrient-rich plates are used, as this inhibits the growth of oligotrophs. Additionally, there may be metabolic obstacles for organisms like obligate syntrophs, in which a bacterium needs a partner to grow. It is possible to predict solutions to these obstacles using metagenomic data, but even with these genomic clues, there are emergent properties that are apparent when studying the whole organism that can't be predicted from genomics alone. Additionally, annotation databases are notoriously incomplete and inaccurate, so isolates are needed for studies to fill in these gaps.

Obtaining isolates of interest from a specific environment can be particularly helpful to further understand communities of interest such as the human gut microbiome, whose members contain probiotic properties, and soil microbiomes that contain Plant Growth Promoting Microbes (PGPM). While many studies have isolated microbes and demonstrated putative PGPM activity *in vitro* or laboratory settings (Souza, Ambrosini, and Passaglia 2015), there has been limited success translating these discoveries into field-based applications. This difficulty is in part due to the poor understanding of underlying mechanisms these microbes utilize to improve the growth of their host plant, the ecology of these organisms, and the interactions between individual microbes and the community at large. For example, it is possible that interactions between PGPM and other members of the rhizosphere community can affect

whether they produce the necessary molecules or phenotypes that benefit the plant. To better understand PGPM and to use them in field applications, it is critical to have cultivable isolates in hand. There is evidence that *Actinobacteria* are beneficial for plant hosts during drought (Xu et al. 2018; Hamedi and Mohammadipanah 2015), though many mechanistic details remain unclear. For example, it is unclear what signals produced by the plant attract *Actinobacteria* during drought, how *Actinobacteria* perceive these signals, how *Actinobacteria* evade the host immune system, and what mechanisms are being used by individual isolates to confer benefits to the host. To investigate these questions, we sought to build an *Actinobacteria*-rich strain collection of *Sorghum bicolor* (sorghum) root endophytes.

Having a strain collection of this nature derived from droughted sorghum roots is valuable because it allows us to study bacteria that were isolated from our environment of interest, rather than obtaining isolates from other collections that likely did not originate from sorghum roots. This is important because microbial species originating from different environments can vary widely in their accessory or pan-genomic content (Silby et al. 2009). Another goal for the isolates in this strain collection is to amass a collection of assembled genomes, so we can identify genes associated with isolates that are PGPB. However, previous attempts to sequence *Actinobacteria* genomes have been limited because they possess relatively large genomes (10 Mbp), have high GC content (over 70% in some cases), and have a high number of repetitive elements (Gomez-Escribano, Alt, and Bibb 2016; Ventura et al. 2007). Additionally, the extraction of high molecular weight gDNA is not trivial for many *Actinobacteria*, as their peptidoglycan-rich cell walls are resistant to cell wall digestion.

Herein, I describe the construction of a large strain collection of sorghum endophytes using two methods, both of which were performed with the intention of enriching for *Actinobacteria*. The first isolation method I used is the long-established technique of hand picking colonies from agar plates, where *Actinobacteria* can be separated using visual and tactile clues. Secondly, I established a new high-throughput bacterial isolation process for root endophytes using a robotics system called the Prospector™, which cultivates tens of thousands of isolates in microwells prior to transferring to a 96-well plate. The end result was a collection of approximately 400 hand-picked isolates and 1500 from the Prospector™.

3.2 Results

3.2.1 High-throughput robotics yield a large, targeted strain collection

We sought to build an isolate library of diverse endophytic *Actinobacteria* that would be used to study how these species interact with and potentially improve the growth of their plant hosts during drought. One library, collected by plating the root material of drought-stressed sorghum onto petri dishes with various media types (Figure 1), yielded 407 isolates. Of these, 354 were able to be classified to the genus level by sequencing the full length 16S rRNA gene using universal primers. These strains represent 36 unique genera that fall within the phyla: *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* (Figure 2, Table 1). As a result of the targeted isolations, 241 of the identified strains (68%) were *Actinobacteria*. Of those, 187 were *Streptomyces*, and the remaining *Actinobacteria* represent 14 different identified genera.

In an effort to improve the speed of isolating environmental bacteria, we collaborated with the company GALT, Inc. to adapt their Prospector™ technology for compatibility with root samples. The Prospector™ is a robotics workflow that loads an environmental sample with growth media and a colorimetric growth indicator onto a microarray (25 mm x 75 mm) with over 6000 microwells (3 nL volume). The sample is loaded such that each well contains either zero or one cell, so any wells with growth contain pure cultures. Using the Prospector™, we isolated 1490 strains of endophytic bacteria. Of these, 617 have been classified. There are 25 unique genera from the phyla: *Firmicutes*, *Actinobacteria*, and *Proteobacteria* (Figure 3, Table 1). Using media that was previously demonstrated to be successful in isolating *Actinobacteria* from plates (Table 2), we were able to obtain a similar distribution of *Actinobacteria* (64%) in the overall collection (Table 1). Notably, this work was performed in only 10 weeks, while the continuous work of hand-picking was done over 2 years.

3.2.2 Media types behave differently between two workflows

When comparing the four media types that were used in both workflows (ISP2, Humic Acid, M9 minimal media, and Tap Water Yeast Extract), we see that there are differences in the percentage of obtained isolates that are *Actinobacteria* between the hand-picked and the Prospector™ methods (Figure 4). This resulted in an overall difference in diversity between the media types on different platforms. The only media that yielded a higher percentage of *Actinobacteria* using the Prospector™ compared to hand-picking was the nutrient-rich ISP2 media. While the distribution of *Actinobacteria* isolated with the Prospector™ varied between media types, the percentage collected using rich media was the closest to the natural endophyte community (Figures 4, 5). It is also interesting to note that most of the *Actinobacteria* acquired through hand-picking were *Streptomyces*, while those collected through the Prospector™ were mostly *Microbacterium* and *Mycobacterium* (Figures 2, 3).

3.3 Discussion

3.3.1 High-throughput technologies can speed up collection of environmental isolates

Isolating bacteria from environmental study systems by picking colonies from plates is a time, labor, and material-intensive process. By using the Prospector™ system, we were able to collect over three times as many isolates in one tenth of the time (Table 1). Additionally, our goal was to build a library abundant in *Actinobacteria*. In this regard, the Prospector™ workflow yielded a slightly lower percentage of *Actinobacteria* (64% compared to 68%). However, the total number of *Actinobacteria* obtained was greater and obtained more quickly. In contrast to hand-picking, the time-limiting step for the Prospector™ system is identifying the isolates. We obtained nearly 1500 isolates, but only have genus identities for 617 of them.

While the clearest benefit of the Prospector™ is the time saved, there are additional benefits to using the system compared to hand-picking colonies. First, the Prospector™ uses far fewer materials, since there is no need for thousands of petri dishes, and only a few milliliters of media are needed for a single microarray, until isolates are transferred into 96-well plates. Without the need for a plethora of agar plates, the amount of space required using the Prospector™ is significantly reduced. By saving space, materials, and time, the Prospector™

system ends up saving cost as well. Additionally, the use of the machine removes unconscious bias and subjectivity that hand-picking by humans may be subject to.

3.3.2 *Actinobacteria*-media type preferences differ between plates and the Prospector™

The only media that we tested that was better at isolating *Actinobacteria* preferentially was ISP2. As rich media, it is difficult to isolate slow-growing bacteria using this media on plates, as they are quickly overtaken by fast-growing colonies. With the Prospector™ platform, individual bacterial cells are incubated in separate wells, so the slow-growing bacteria are allowed to grow unimpeded. For other media (Skim Milk, M9 minimal media, and Tap Water Yeast Extract), the percentage of isolates that were *Actinobacteria* decreased when using the Prospector™. It is possible that while *Actinobacteria* grow well on these media (as evidenced by hand-picking), these media may alter *Actinobacteria* morphology in such a way that they are incompatible with the Prospector™, and unable to be transferred from the Prospector™ microwells to the 96-well plate for further growth. For example, there is evidence that bacteria may alter the shape of their cell wall or form spores in nutrient limiting conditions (van Teeseling, de Pedro, and Cava 2017). Since the Prospector™ relies on physical adhesion of cells to a needle to transfer them to the 96-well plate for growth, it is not unreasonable to hypothesize that certain cell morphologies would not be transferred.

At a genus level, we see a distinction between the isolates obtained using the two methodologies. Difficult cell morphologies could be a reason why so few *Streptomyces* were isolated using the Prospector™. *Streptomyces* likely dominated our hand-picked isolate collection for two reasons. First, this genus is known to synthesize potent antibiotics, which could kill other colonies growing on the same plate (de Lima Procópio et al. 2012). Additionally, the physical characteristics screened for were all derived from *Streptomyces*. Spores, colors indicative of secondary metabolites, and an "earthy smell", are all phenotypes we picked for that describe *Streptomyces*, but not all other *Actinobacteria (Hasani, Kariminik, and Issazadeh 2014)*. The dominant genera in the Prospector™ library were *Microbacterium* and *Mycobacterium*, which perhaps do not frequently display these characteristics.

3.3.3 The importance of specialized strain collections

Development of publicly available strain collections can be beneficial to microbiologists across subdisciplines. While it may be sufficient to obtain a small number of strains from an existing collection such as ATCC or the USDA, it may be better to have microbes that originate from a particular environment. For environments such as the root endosphere, having a strain collection is particularly valuable, because shotgun metagenomics efforts are hindered by the large amount of host DNA (Lucaciu et al. 2019).

While amplicon studies profiling the 16S rRNA gene are valuable for discovering what bacteria are present in an environment, only a limited amount of microbial community function can be inferred using this method (Langille et al. 2013). In contrast, having a strain collection would allow microbiologists to sequence the genomes of organisms of interest, where function can be more readily predicted. For example, these genomes could be used in a comparative study

to look for genomic clues for why certain bacteria persist in environments such as plant roots (Levy et al. 2017). An additional benefit to having an isolate collection with sequenced genomes rather than performing shotgun metagenomics is the ability to validate available annotations.

In addition to validating functionality for the purposes of improving annotation databases, we can also use isolates in experiments to understand interspecies interactions. The mechanisms behind many PGPB have been discovered using experiments with isolates in the lab (Souza, Ambrosini, and Passaglia 2015). Isolates are also useful for studying the interactions between bacteria within an environment, as a means to understanding the ecology of a system. Building synthetic communities (SynComs) of microbes isolated from the same environment (as shown in Chapter 4) can provide valuable insights into community characteristics such as growth and structure, dominance/ keystone species, succession, and potentially trophic structure.

3.4 Materials and Methods

3.4.1 Plant growth and tissue collection

For all isolations, sorghum plants were grown at the University of California's Agriculture and Natural Resources Kearney Agriculture Research and Extension Center in Parlier, CA, as described previously (Xu et al. 2018; C. Gao et al. 2020). Root samples were obtained from mature sorghum plants that had been subjected to a prolonged pre-flowering drought. Immediately after extraction of plants from soil, roots were removed and placed in 25% glycerol for 30 mins, then placed on dry ice until they were transferred to -80°C. To remove soil, roots were placed in a phosphate buffer and sonicated briefly. They were subsequently vortexed for 60s in 99% ethanol, 6 mins in 3% NaOCl, and 30s in 99% ethanol to sterilize the root surface. Roots were washed twice in sterile deionized water, and 100 μL of rinse water was plated to check surface sterility. Roots were then cut into 1 cm pieces and placed into 2 mL tubes with 25% glycerol and incubated for 30 mins at room temperature before storing at -80°C. A visual representation of this workflow is shown in Figure S1.

3.4.2 Isolation of *Actinobacteria* by picking from plates

One 2 mL tube of roots (approximately 200 mg) was thawed and placed in a sterile ceramic mortar with 1 mL PBS buffer. Root tissue was ground gently, to release endophytic bacteria into the solution while minimizing lysis of bacterial cells. The solution was serially diluted, and 100 μL of dilutions 10^{-1}, 10^{-2}, and 10^{-3} were plated onto various media types: ISP2, M9 minimal media, Skim Milk, Tap Water Yeast Extract, and Humic Acid (recipes in Appendix B). Plates were placed at 30°C and growth monitored daily. When colonies were visible, they were picked and streaked onto a fresh plate of ISP2, followed by subsequent streaks if necessary to eliminate contamination, until only a single morphology was observed.

To maximize the selection of colonies likely to be *Actinobacteria*, we prioritized colonies possessing the following characteristics common in representatives of this lineage: production of spores, antibiotics (suggested by the presence of a red, orange, yellow, or blue color), stiff surface texture, matte surface color, amorphous edges, ring shaped colonies, and/or a 'wet earth' smell (suggestive of geosmin production).

3.4.3 Isolation of *Actinobacteria* using the Prospector™

When conducting isolations using the Prospector™ technology, one 2 mL tube of roots (approximately 200 mg) was thawed and ground either using a sterile ceramic mortar and pestle or using stainless steel grinding units of a Qiagen TissueLyser II (ground at 30 Hz for 3.5 minutes). In either case, roots were ground with the PBS buffer. Particles from the root-media slurry were removed using several methods: allowing the particulate matter to settle, gentle centrifugation, filtering with a sterile cheesecloth, and/or filtering with a 10 μm filter. Bacterial cells were pelleted by centrifuging at maximum speed for 10 minutes, and the pellet resuspended in 10 mL of culture medium.

Cell suspensions with media were mixed with the growth indicator resazurin and then loaded onto the Prospector™ microarray (6109 wells) following manufacturer's instructions. Four media types were used: ISP2, M9 Minimal Media, Skim Milk, and Tap Water Yeast Extract (recipes in Appendix B). Growth was monitored each day by imaging on the Prospector™ by measuring fluorescence under red and green wavelengths and calculating the ratio between the two. Wells with growth after 7-10 days, as determined by a shift in fluorescence ratios, were transferred into 96-well plates containing either ISP2 or LB liquid media.

3.4.4 DNA extraction and identification of isolates

For all isolates, an ID was obtained by performing colony PCR on a culture. Cells were lysed by incubating 6 L of culture with 10 μL of lysis buffer (25 mM NaOH + 0.2 mM EDTA, pH 12) at 95°C for 30 mins. The pH was lowered by adding 10 μL of 40 mM Tris-HCl, pH 7.5. This was used as the template (1.5uL) for a PCR reaction amplifying the 16S rRNA gene (universal primers 27F and 1492R). Clean PCR product was Sanger sequenced using one of the primers. For a portion of the isolates obtained with the prospector, culture plates were sent to either Genewiz or Loop Genomics to perform the colony PCR and sequencing protocols. Taxonomic classifications of isolates were obtained from sequences using the top match in a Basic Local Alignment Search Tool (BLAST) search.

3.4.5 Sequence alignment, tree building and visualization

16S sequences were aligned and phylogenies constructed using SILVA's ACT (Alignment, Classification, and Tree Service) online tool (Pruesse, Peplies, and Glöckner 2012). We used a representative 16S sequence for each of the genera present in the strain collection (chosen by the longest sequence with the lowest percentage of unknown bases). The search and classify parameters were: 0.98 minimum identity with query sequence, and 10 neighbors per query sequence. Trees were computed using the RAxML program, the wordflow "denovo including neighbors", the GTR model, and the gamma rate model for likelihoods. The trees were then downloaded and imported into the iTOL (International Tree of Life) online tool for visualization and the addition of metadata (Letunic and Bork 2019). Graphics in this study were created using BioRender.

Chapter 3 Main Figures

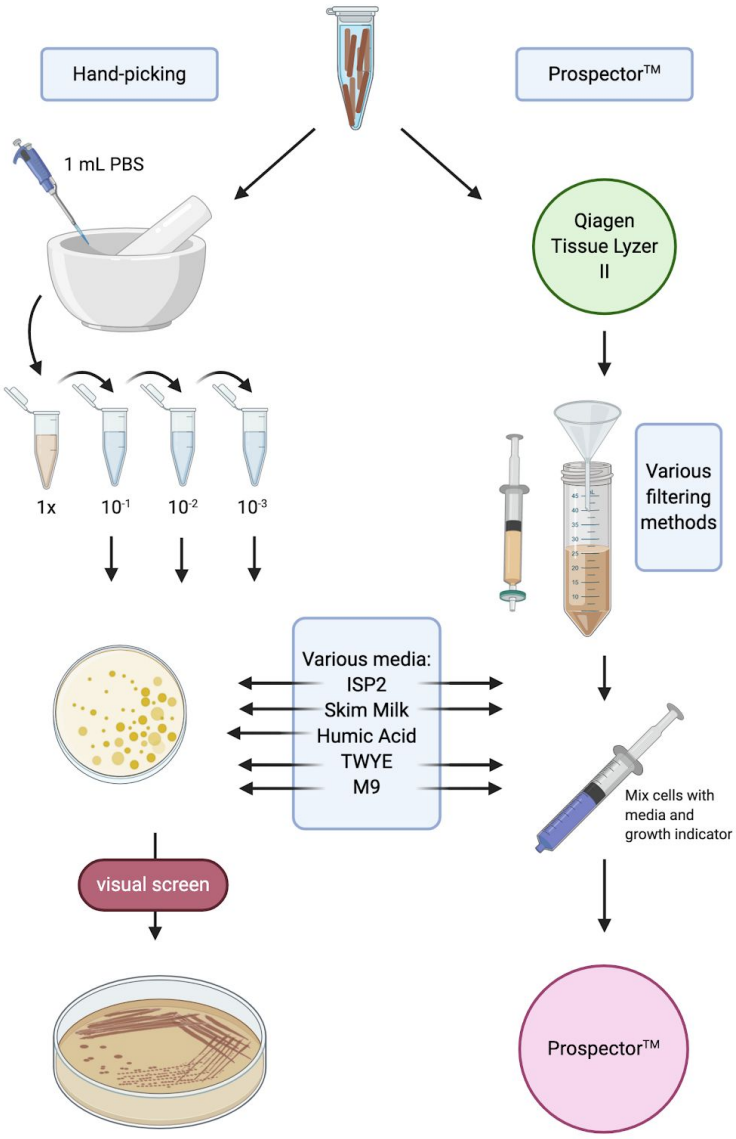

Figure 1.
Diagram comparing the two workflows used for collecting bacterial isolates from drought-stressed sorghum roots.
Created using BioRender.

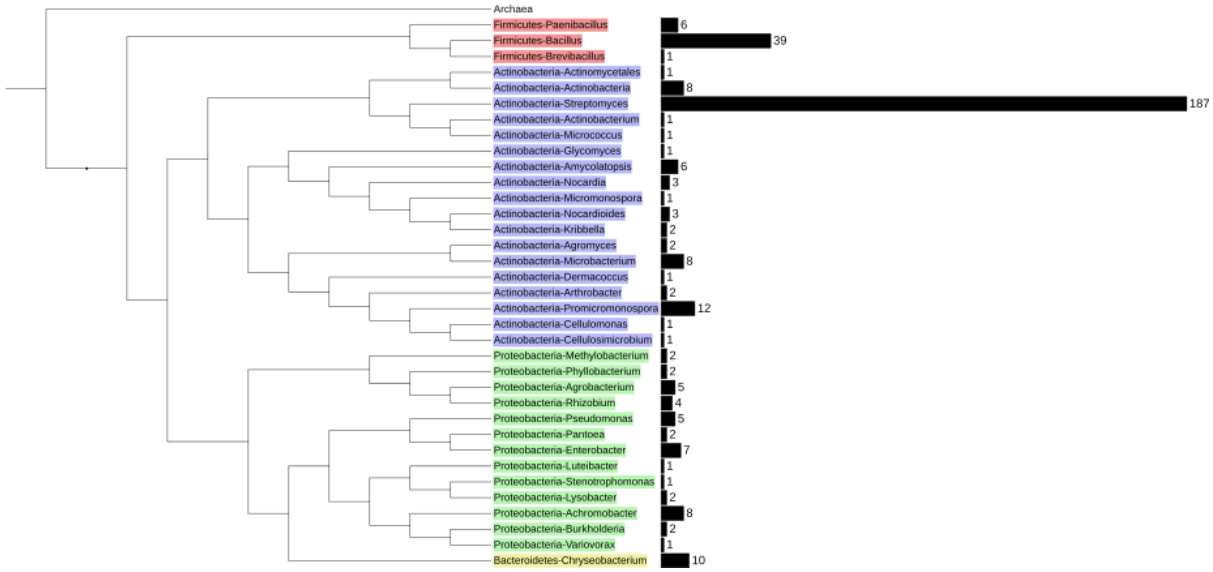

Figure 2.
Phylogenetic tree of genera present in the original strain collection of isolates that were collected by picking colonies from agar plates. Tree was constructed using a representative 16S sequence from each genera and an Archaea sequence to root the tree, and input into SILVA's ACT online tool. Tree was visualized using the iTOL online tool. Bars and numbers represent the number of strains from that genera that are present within the collection.

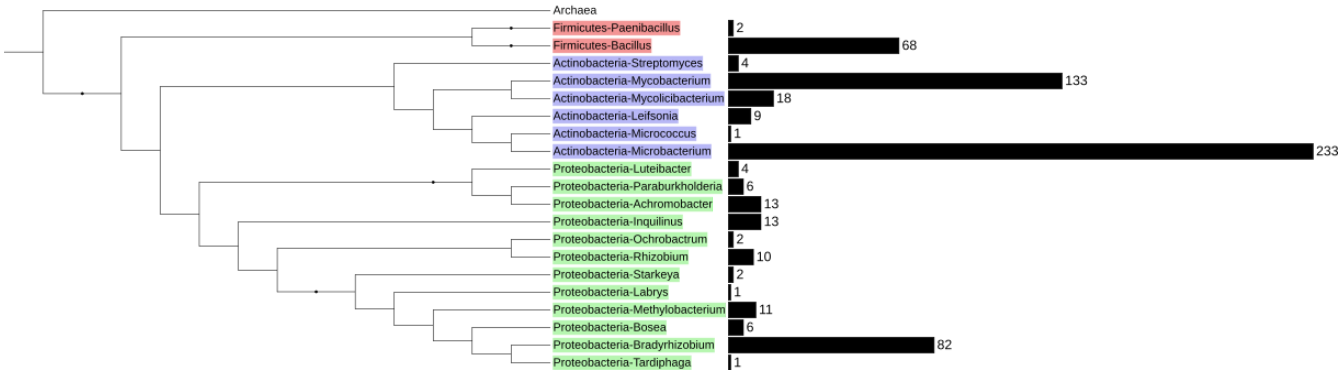

Figure 3.
Phylogenetic tree of genera present in the strain collection of isolates that were collected using the Prospector™ platform. Tree was constructed using a representative 16S sequence from each genera and an Archaea sequence to root the tree, and input into SILVA's ACT online tool. Tree was visualized using the iTOL online tool. Bars and numbers represent the number of strains from that genera that are present within the collection.

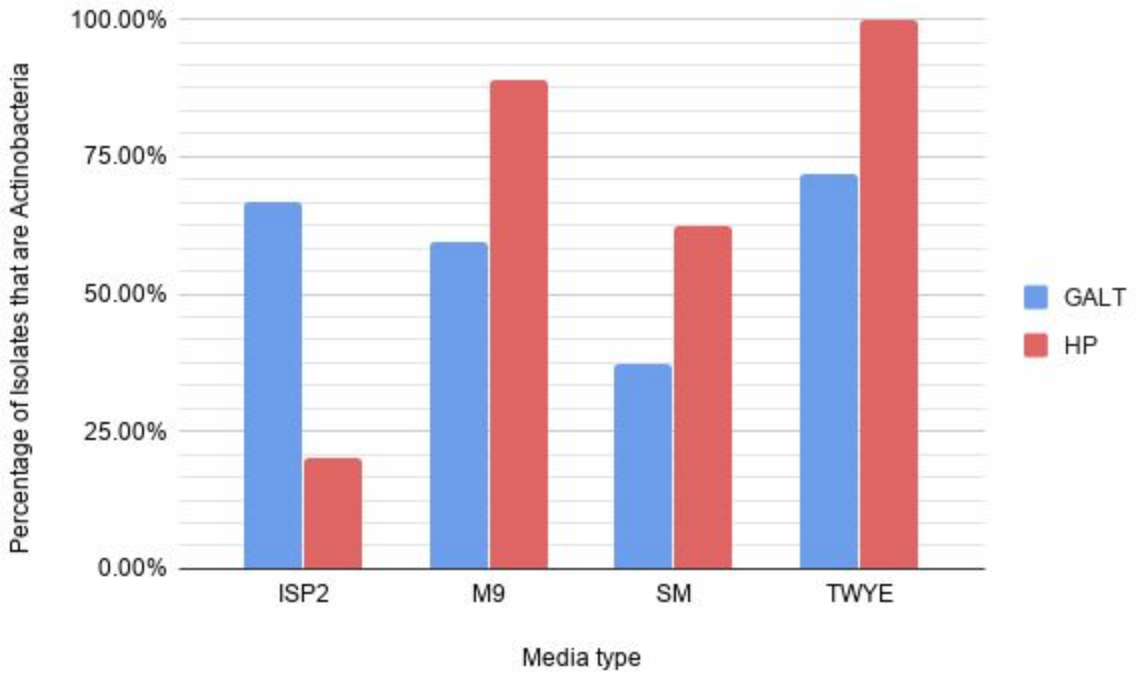

Figure 4.
Percentage of isolates obtained from four media types that were *Actinobacteria*. Blue bars indicate isolates that were obtained using the Prospector™ (GALT, Inc.) and red bars indicate isolates that were selected as colonies from agar plates (HP). Values can be found in Table 2.

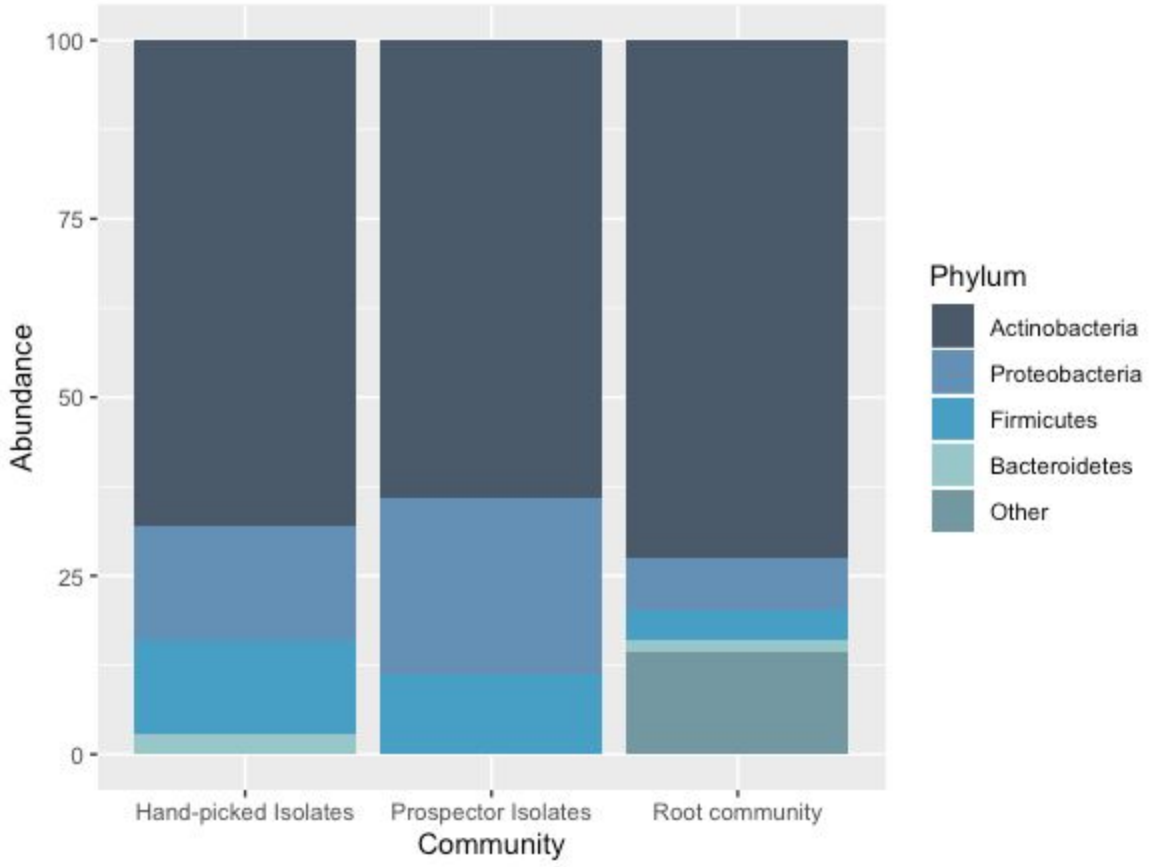

Figure 5.
Relative abundance of the four isolated phyla in the two strain collections and in a sorghum root endophyte community. Abundance in the strain collections is measured by the number of strains collected in each phylum. In the root community, abundance is determined by read counts.

Table 1.
Comparison of strain collections collected using the two different workflows (picking colonies by hand or the Prospector™ system).

	GALT	Hand-picked
Number of ID'd isolates	617	354
Number ID'd as *Actinobacteria*	396	241
% of ID'd as *Actinobacteria*	64.18%	68.08%
Number of genera	25	36
Time spent collecting (months)	2.5	24

Table 2.
Comparison of different media used in the two different workflows (picking colonies by hand or the Prospector™ system) and their yield of *Actinobacteria* (Actino).

	Media	***Total***	***Actino***	***% Actino***
Hand-picked	ISP2	5	1	20.00%
	M9	9	8	88.89%
	SM	80	50	62.50%
	TWYE	33	33	100.00%
	SCA	57	41	71.93%
	AIA	64	41	64.06%
	HA	32	21	65.63%
	HA5.5	37	31	83.78%
	Unknown	37	12	32.43%
GALT	ISP2	154	103	66.88%
	M9	136	81	59.56%
	SM	67	25	37.31%
	TWYE	260	187	71.92%

Chapter 3 Supplemental Figures

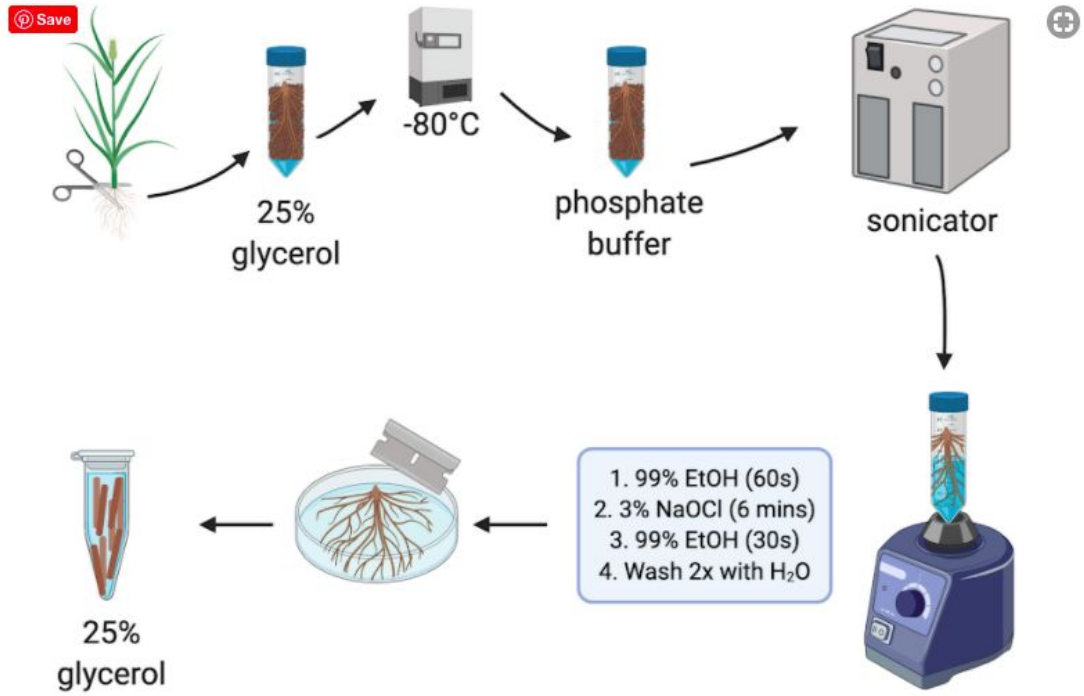

Figure S1.
Workflow for the collection and surface sterilization of root tissue to be used for isolating endophytic bacteria. Created using BioRender.

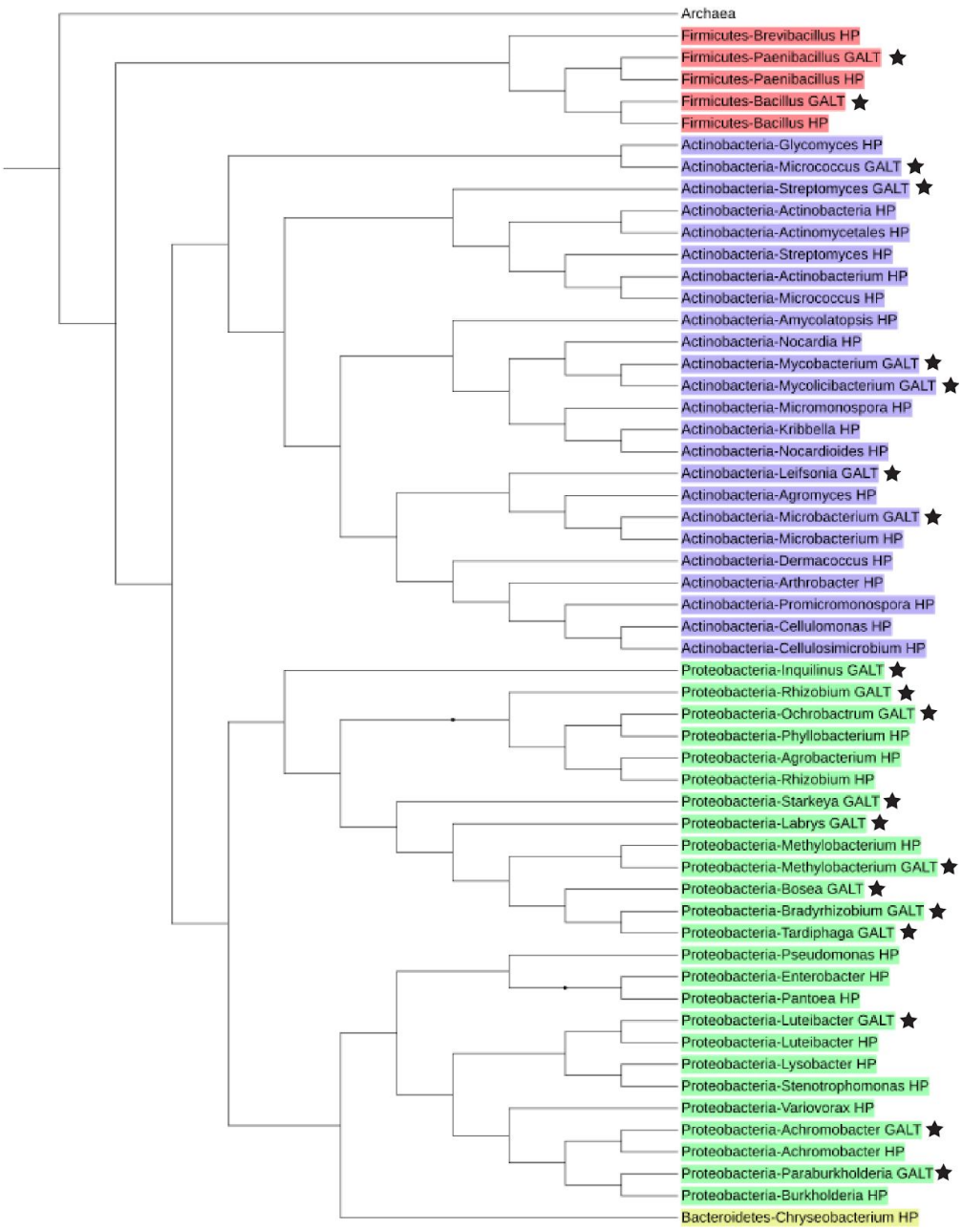

Figure S2.
Phylogenetic tree of all genera present in the strain collections of isolates that were collected using hand-picking from agar plates and the Prospector™ platform. Tree was constructed using a representative 16S sequence from each genera and an Archaea sequence to root the tree, and input into SILVA's ACT online tool. Tree was visualized using the iTOL online tool. Bars and numbers represent the number of strains from that genera that are present within the collection. Stars indicate isolates collected using the Prospector™.

Chapter 4: Using Synthetic Communities to Understand the Role of Endophytic *Actinobacteria* in Promoting Plant Growth During Drought Stress

4.1 Introduction

In the field of macroecology, it is relatively easy to observe interactions between different species in their natural environments. A lynx eating a hare (predation), a clownfish living in sea anemone (mutualism), and a deer tick living on a dog (parasitism) are all examples of symbioses that can be observed and classified with a high degree of confidence. In contrast, drawing conclusions about interspecies interactions in a natural microbial community is much more difficult. Many of these interactions are mediated by chemical signals, so they are not observable under a microscope. Using tools like metagenomics and metabolomics in conjunction with modelling tools such as co-occurrence networks, we are able to generate predictions about what species are interacting and what type of interactions are occurring. However, additional evidence is required to determine if these interactions are true. In order to verify these predictions, it is best to have cultivable isolates in hand. It is also preferable that these isolates were obtained from the same environmental background.

Microorganisms have long been used by humans as remedies for various maladies in the form of probiotics (Fuller 1989). While this application is most well-studied in the context of the human gut microbiome, humans have been unintentionally seeding soil communities with probiotics for millenia in the form of compost (Diaz and de Bertoldi 2007). As our understanding of beneficial microbes for both human health and agriculture has expanded, studies of single-strain inocula have dominated (Souza, Ambrosini, and Passaglia 2015). These individual strains are classified as PGPM - Plant Growth Promoting Microorganisms. Unfortunately, the application of an individual PGPM to an already stable microbial community is unlikely to result in the long-term incorporation of the applied strain (Shade et al. 2012). This means that the timing of these PGPM must be carefully considered, and increases the likelihood that these PGPM will necessitate multiple applications. In agricultural systems, there are various strategies that can be used to apply PGPM. They may be applied to either the soil or the plant (seed or stem cutting), as either a liquid or dry inoculum, at the seed or seedling stages (to increase establishment) or during a growing season (in anticipation or in response to a potential stress) (Souza, Ambrosini, and Passaglia 2015; Malusá, Sas-Paszt, and Ciesielska 2012).

PGPM inoculants are able to improve the growth of their plant host in several different ways. Some function as biofertilizers - assisting in nutrient solution from the surrounding soil. For example, bacteria can help solubilize phosphate, fix nitrogen, and uptake iron. Some species synthesize or modulate levels of plant growth hormones, such as indole-3-acetic acid and ethylene. Other PGPM are classified as biopesticides, or biocontrol agents, and function by protecting against plant pathogens. Recent studies have shown that strains of *Actinobacteria* act as PGPM during drought (Xu et al. 2018), though the mechanism has yet to be uncovered. While previous experiments with *Actinobacteria* have used single-strain inocula, we propose the application of *Actinobacteria* PGPM as members of stable synthetic communities in agricultural settings to help alleviate drought stress.

Microbial communities can be considered stable if they are both resistant and resilient to disturbances (Bryan S. Griffiths and Philippot 2013). Properties that contribute to community stability include: intra- and interspecies genetic diversity (Heuer, Abdo, and Smalla 2008; Erkus et al. 2013), variation in positive and negative interactions between community members and the strength of those interactions (Coyte, Schluter, and Foster 2015), and species richness (Girvan et al. 2005; Shade et al. 2012). In this study, to create the most stable synthetic communities, we made predictions for interactions by building co-occurrence networks from existing data derived from plant roots during drought (Xu et al. 2018).

In a network, nodes represent microbial taxa where edges represent significant correlations between taxa. While we cannot assume that all connections within a network represent real interactions (Berry and Widder 2014), co-occurrence networks can be used to build hypotheses about interactions between members of microbial communities. Previous studies using network analysis to study microbiomes have shown that: modules within networks can indicate ecological processes (Lima-Mendez et al. 2015), host plants manipulate their microbiomes through "hub" microbes (Agler et al. 2016), and soil bacterial communities are less stable than fungal communities during drought (de Vries et al. 2018). In this study, we use the hypothesized interactions from the co-occurrence network to build synthetic communities.

Using the co-occurrence network, we created five different 16 member synthetic communities (SynComs), to allow for a sufficient but manageable level of diversity. These SynComs consisted of 100%, 75%, 50%, 25%, or zero *Actinobacteria*, while the other community members were from the other three most dominant phyla in endophyte communities: *Proteobacteria, Firmicutes,* and *Bacteroidetes.* Additionally, we used isolates from our own strain collection that originated from drought-stressed roots of *Sorghum bicolor* (sorghum), so each community member came from the same environmental background. After applying the SynComs to gnotobiotic sorghum, we found that the only community that provided a benefit during drought was one that contained only *Streptomyces*.

4.2 Results

4.2.1 Design of SynComs using co-occurrence networks

In this study, our goal was to test whether the abundance of *Actinobacteria* in SynComs impacted the efficacy of the community's plant growth promotion (Supplemental Figure 1). We constructed five 16-member communities with varying abundances of *Actinobacteria* (from 100% to 0%, in increments of 25%). We also constructed a 16-member community without any *Firmicutes*, another gram-positive phyla that is enriched during drought, and one community that consists only of *Streptomyces*, a genus of known PGPB.

In an effort to design SynComs that would be stable communities, we used an existing dataset (Xu et al. 2018) to predict interactions between bacterial root endophytes by building a co-occurrence network. Using 16S amplicon community data from drought-stressed root endosphere samples, we used SparCC to calculate a correlation matrix. Correlations with coefficients between 0.6 and -0.6 were removed, thus the remaining correlations were those of

the most positive and most negative values. This resulted in a network with 113 nodes (ASVs) (Figure 1). The partial 16S sequences for these ASVs were compared against a local database of 16S sequences from an in-house strain collection to identify the strain most closely related to each node. All strains present in the SynComs came from this collection, so they all have the same environmental history (drought-stressed sorghum root endosphere). The final list of community members can be found in Table 1.

4.2.2 Normalization of SynCom inoculum by biomass does not generate an even community

In order to apply equal amounts of each strain present in a SynCom, we decided to normalize cell density by biomass. While using OD measurements is ideal, the *Actinobacteria* used in our SynComs grow in clumps in liquid culture, making it impossible to measure OD. SynComs were applied by adding normalized mixtures to a nutrient solution, which was added to sterilized calcined clay inside a sterilized 5L plastic growth environment. The remaining inoculum for each SynCom was pelleted, DNA extracted, and the community profiled using the same amplicon sequencing workflow that was used for the root samples.

If all strains have the same biomass density, sequencing the inoculum would show concentrations of each genera proportional to the number of strains within that genera (Figure 2). This theoretical starting abundance also takes into account the predicted number of copies of the 16S rRNA gene each strain has (Stoddard et al. 2015). When the inoculum is sequenced, we see significantly fewer *Actinobacteria* and *Firmicutes* reads than expected (Figure 2). There are two possible explanations for this. First and foremost, it is possible that the density of these two phyla are higher than *Proteobacteria* and *Bacteroidetes* due to the production of more exopolysaccharides or other organic molecules. A second explanation that we know to be true, is that extraction of mixed communities is biased against gram positive bacteria (Roopnarain et al. 2017; Teng et al. 2018).

4.2.3 SynComs are able to colonize roots

Despite the possibility that the inocula, although replicable, were unevenly distributed (Figure 2), we found that after five weeks of plant growth, representatives from each of the genera within the SynComs were found to colonize the roots (Figure 3). We also see that in the blank samples (roots grown under the same conditions with no bacterial inoculum), there is a seed endophyte, Erwinia, that is also present in the roots of five different treatments. It is interesting to note that in all of the SynComs that had *Actinobacteria*, the final relative abundance of *Actinobacteria* is similar (80%) (Figure 3).

4.2.4 A SynCom containing only *Streptomyces* was most effective at promoting plant growth

Plant material was harvested after five weeks of growth, and the fresh shoot and root biomass was measured (Figure 4). While an attempt was made to impose a drought stress, there was not a significant difference in soil moisture between the two watering treatments (31% for control, 27% for “drought”). When comparing the biomass measurements of SynCom treated plants to the gnotobiotic “blank” plants (no bacterial inoculum), the only measurement that showed a statistical difference ($p<0.05$, Wilcoxon test) was an increase in shoot biomass of the *Streptomyces* SynCom treated plants (Figure 4).

Because of the lack of drought stress, we decided to repeat the experiment with a few modifications. We set up the experiment in the same way and allowed the plants to grow for two

weeks before collecting an initial time point. After two weeks, the lids maintaining the sterile environment were removed from ⅔ of the boxes for each SynCom treatment. For the remaining ⅓, a heightened environment was constructed to maintain sterility (Supplemental Figure 2). Half of the boxes without lids were watered, while the other half were allowed to dry out to induce a drought stress. After three weeks of growth in this manner, plant material was collected. At this time, only fresh shoot biomass has been measured due to COVID-19 laboratory entry restrictions. From this data, we see that no SynComs altered shoot biomass when plants were grown in a well-watered environment. When plants were drought stressed, two communities promoted shoot growth: *Streptomyces* only, and 25% *Actinobacteria* ($p<0.05$, Wilcoxon) (Figure 5).

4.3 Discussion

4.3.1 Using networks to design SynComs may improve stability

If the goal of SynComs is to study the interactions between bacterial species and understand the ecology of microbial communities, the construction of these communities should be well-reasoned and thought out. In this study, we decided to build SynComs with 16 members because it introduces sufficient complexity, yet not more than can be reasonably grown in high volume at one time in a laboratory setting. Additionally, we used an in-house strain collection in order to ensure that all SynCom members came from the same environmental background. Since we know that variation in positive and negative interactions within a community can increase the stability of that community (Coyte, Schluter, and Foster 2015), we decided to use hypothesized interactions (co-occurrence networks) to build our SynComs.

From the evidence that has been collected thus far, we have reason to believe that our network-generated SynComs have some degree of stability, as they are able to successfully colonize the roots of sorghum plants grown in a gnotobiotic environment and persist for at least the length of time used in this study. However, additional experiments should be conducted in the future to determine the stability of the SynComs. First, amplicon sequencing should be performed for the SynComs in the follow-up experiment in which we have plant phenotypic data, in order to verify that the SynComs were successful in colonizing roots. Additionally, since the sterile environment was disrupted in this experiment, we should determine whether the SynComs were resistant to invasion by any potential contaminants. Second, future experiments can be performed where the SynComs are applied in a field setting, to test how well the communities are able to invade and persist in an existing community.

4.3.2 Plant growth promoting properties may differ as a result of interspecies bacterial interactions

Although we have labelled certain microorganisms as Plant Growth Promoting Microbes (PGPM), it should be noted that the mechanism by which they benefit plants may not be constitutively expressed. For example, many antibiotics produced by *Actinobacteria* that may fight off plant pathogens are only expressed under certain conditions (Behie et al. 2016). Even more importantly to consider when applying engineered communities to natural environments, the same microorganism may be beneficial to one host plant and pathogenic to others or under

certain conditions. In our study, we have observed that a SynCom containing only *Streptomyces* is effective at increasing the biomass of drought-stressed sorghum, while a SynCom that contains these same species in addition to 11 other *Actinobacteria* (100% Actino, Table 1) was not effective. This suggests that the presence of one or more of the additional species in the 100% Actino community causes one or more of the *Streptomyces* to cease production of the plant-growth promoting molecule or mechanism. Importantly, these SynComs did not reduce plant growth in watered conditions. Future research is needed to determine which member(s) of the *Streptomyces* SynCom is a PGPB, what mechanisms they are using, and why the addition of additional community members inhibits this activity.

4.4 Materials and Methods

4.4.1 Constructing a bacterial co-occurrence network

Amplicon data from a previously published dataset (Xu et al. 2018) was subsetted and used to construct the co-occurrence network. Briefly, for samples used in the network: Sorghum was grown for 6, 7, and 8 weeks at the University of California Kearney Agricultural Research and Extension Center in Parlier, CA. The plants were watered for the first three weeks, and water was withheld for the next five weeks to induce a drought stress. Root sampling, DNA extraction, and amplicon sequencing were performed as described previously (Xu et al. 2018). In brief, root samples were collected and the rhizosphere washed off with a phosphate buffer. Bacterial community profiling was performed by amplifying the v3-v4 region of the 16S rRNA gene and sequencing in a barcoded library on the Illumina MiSeq platform.

An ASV table containing the samples of interest (drought-stressed root endophytes) was loaded as a text file into python. The table was restricted to the top 500 most abundant ASVs. This was used as an input to SparCC (Friedman and Alm 2012), which provides a correlation matrix as output. In R, the matrix was subsetted to include only the strongest positive and negative correlations (coefficient of greater than 0.6 or less than -0.6). This table was imported into the open-source software Cytoscape (Shannon et al. 2003) to visualize.

4.4.2 Using network to select isolates from strain collection

A local database was created with the 16S rRNA sequences of all the strains in our local isolate collection, described in detail in chapter 3, using blast2go (Götz et al. 2008). The sequences for the v3-v4 region of the 16S rRNA gene were pulled from the nodes of the co-occurrence network (ASVs), and searched against our local database to find the closest matches with isolates available. From this list of isolates, the most abundant of the following were selected: 16 *Actinobacteria*, 12 *Proteobacteria*, 3 *Firmicutes*, and 4 *Bacteroidetes*. These were organized into 7 different SynComs of 16 members each (Table 1).

4.4.3 Normalizing inoculum

Prior to mixing into SynComs, each strain was individually grown in LB broth at 30℃ for 2-7 days until a sufficient amount of biomass was observed, and each strain was transferred to 4℃ for at least 24 hours prior to mixing and inoculation. To ensure that the starting inoculum contained approximately equal amounts of each community member, we normalized each strain by wet biomass. While we recognize the limitations of this method, it was necessary because OD

measurements cannot be made for *Actinobacteria*, which are prone to forming solid clumps when grown in liquid cultures. Once biomass densities were normalized for each strain, they were mixed together in equal amounts.

4.4.4 Growth chamber experimental set-up

The gnotobiotic growth enclosure consists of 1 kg calcined clay (Sierra Pacific Supply http://www.sierrapacificturf.com), 5L clear polypropylene boxes, and lids with a HEPA filter (Combiness, Nevele, Belgium) - all autoclaved sterile (herein referred to as Microboxes). SynComs were mixed into 400 mL of autoclaved sterile 1x Hoagland's nutrient solution and then mixed into the sterile calcined clay in the Microbox using a sterile 50mL serological pipet. Work was done under a laminar flow hood to maintain sterility. Once the substrate was homogenous, 4 sorghum seedlings of equal size (that were surface sterilized for approximately 20 mins with 10% bleach, washed, and placed on sterile plates to germinate at 30℃ for 3 days prior) were placed into the calcined clay and gently covered.

For the pilot experiment, there were 32 Microboxes total. For each SynCom, there were 2 water treatments (control or drought) and 2 replicate boxes. Additionally, there were 2 "Blank" boxes per watering treatment that contained no bacterial inoculum. Control boxes were watered 3 times per week (3 mL sterile water per plant), and lids were removed for watering under a laminar flow hood. Plants were allowed to grow until they were 5 weeks old in a growth chamber kept at 16h:8h Light/Dark, ppf ~120 µmol $m^{-2}s^{-1}$, 33℃/28℃ and 60% humidity.

A larger scale experiment was set up using a nearly identical preparation and input for the growth enclosures, but with the following changes. First, all boxes were grown for 2 week with the lids on before a first time point was collected - 3 replicate boxes per SynCom, containing 4 plants, plus 3 "Blank" boxes (no bacterial inoculum) and 3 boxes with bacteria only (no plants). For the remaining boxes, the lids were removed from 6 of 9 boxes per SynCom treatment, and for the remaining 3, Microboxes were modified such that another sterile Microbox with the bottom cut out was attached, so they could be vertically stacked. This modified setup allowed for plants to continue growing without reaching the lid (Figure S2). These plants were grown for an additional 3 weeks; half of the boxes without lids were watered, and the other half were allowed to dry out to induce drought stress.

4.4.5 Sample collection and phenotypic measurements

After the growth period, plants were harvested individually in a sterile environment. Fresh weight was measured for both roots and shoot biomass. Roots were stored in phosphate buffer and frozen at -20℃ until processed further. Roots were processed by extracting DNA using Qiagen PowerSoil kits (using 150mg ground root tissue). Leftover SynCom inoculum was pelleted and DNA extracted using the PowerSoil kit. Bulk soil was collected from each box for soil moisture measurements. Soil moisture was estimated by measuring the soil wet weight, drying for at least 3 days at 70 °C, and measuring the dry weight to determine the mass of water present.

4.4.6 Community profiling and data analysis

This is derived from methods presented in Appendix A. All samples were amplified in triplicate using barcoded universal primers (180 s at 98 °C, 30 cycles of: 98 °C for 45 s, 78 °C for 10 s, 55 °C for 60 s, and 72 °C for 90 s, then 600 s at 72 °C followed by a 4 °C hold) for the v3-v4 region (341 F, 5'-CCTACGGGNBGCASCAG-3' and 785 R, 5'-GACTACNVGGGTATCTAATCC-3') of the 16S rRNA gene according to (Simmons et al. 2018). Additionally, PNAs matching chloroplast and mitochondrial 16S sequences were spiked into PCRs (2.28 μM final concentration) to prevent amplification of these unwanted reads. Replicate PCR products were pooled and quantified using Qubit HS assay; 100ng from each sample was pooled together and cleaned using AMPureXP magnetic beads before a final quantification and dilution to 10nM for sequencing at the UC Berkeley Vincent Coates Sequence Facility via Illumina MiSeq (v3 chemistry, 300 bp paired-end sequencing). Reads were demultiplexed in QIIME2 (Bolyen et al. 2018) and then passed to DADA2 (Callahan et al. 2016a) where sequences were trimmed to ensure minimum median Phred Q-scores of 30 or greater at any given base pair position prior to denoising and Amplicon Sequence Variant (ASV) inference; 500,000 reads were used to train error-rate models, but otherwise all other pipeline default settings were used. A taxonomy classifier was trained to the V3-V4 region of sequences from the August 2013 version of GreenGenes 16S rRNA gene database via Naive Bayesian methods in QIIME2 and used to assign taxonomic associations to ASVs. All subsequent statistical analyses were completed in R.

Chapter 4 Figures

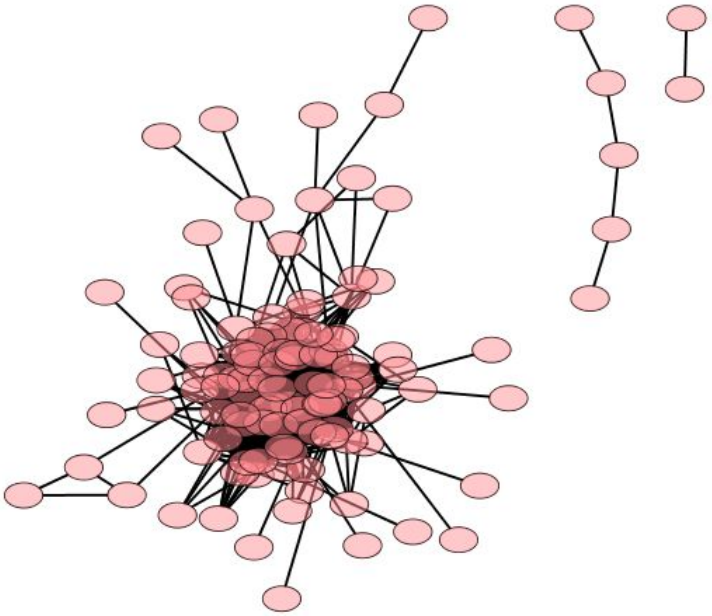

Figure 1.
Co-occurrence network constructed using SparCC to calculate the correlation matrix and visualized using Cytoscape. The correlation matrix was trimmed to include only values > 0.6 and < -0.6. 113 nodes are present in the network that are representative of bacterial ASVs.

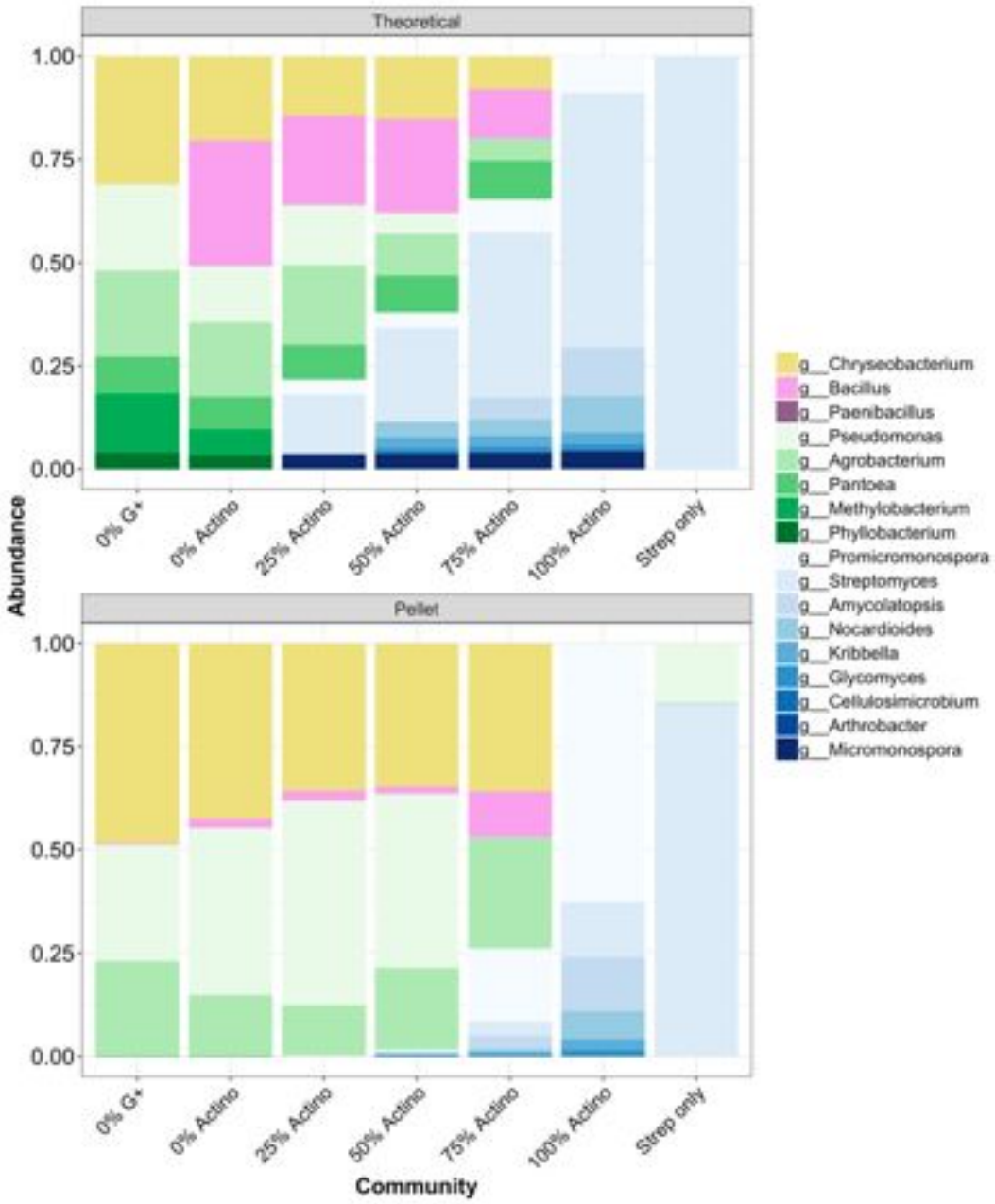

Figure 2.
Predicted read distribution for amplicon sequencing of the v3-v4 region of the 16S rRNA gene of the starting SynCom inocula compared to the actual sequencing results, colored by genus where shades of blue are *Actinobacteria*, shades of green are *Proteobacteria*, shades of pink are *Firmicutes*, and yellow is *Bacteroidetes*. Predicted results (top panel) are based on the number of strains in the indicated genera and the estimated copy number of the 16S gene for that genus.

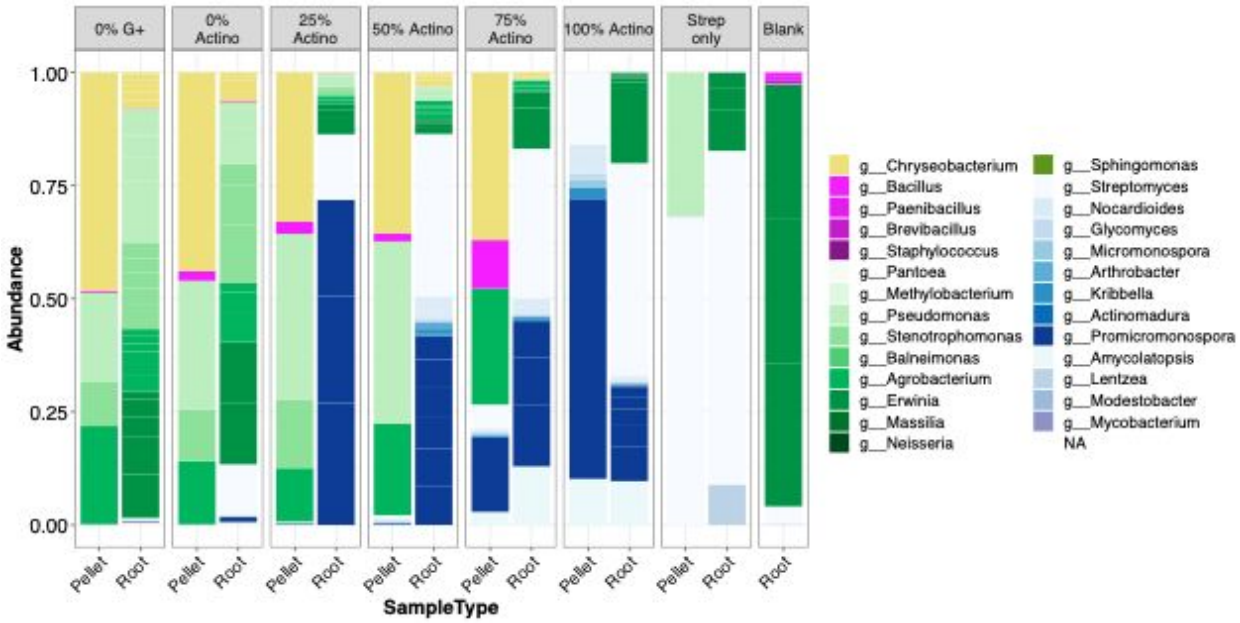

Figure 3.
Relative abundance based on sequence read count of the starting inoculum (pellet) for each SynCom and the root endophyte community after five weeks of growth. Bars are colored by genus where shades of blue are *Actinobacteria*, shades of green are *Proteobacteria*, shades of pink are *Firmicutes*, and yellow is *Bacteroidetes*.

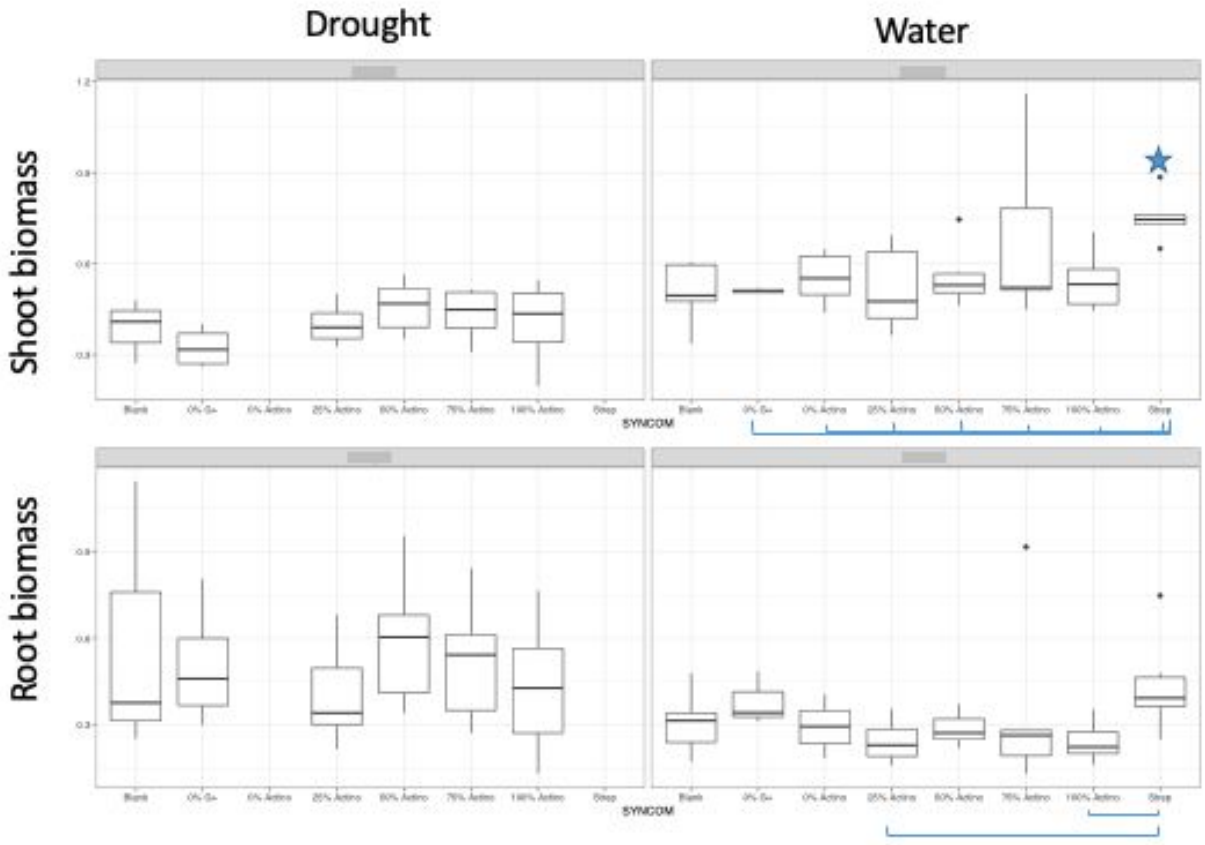

Figure 4.
Root and shoot biomass of plants that survived the pilot SynCom experiment. Center lines in the boxes are means, while the top and bottom lines of the boxes represent the first and third quartile. Star indicates a significant difference from the “blank” plants within the same category, as determined by a Wilcoxon test ($p<0.05$).

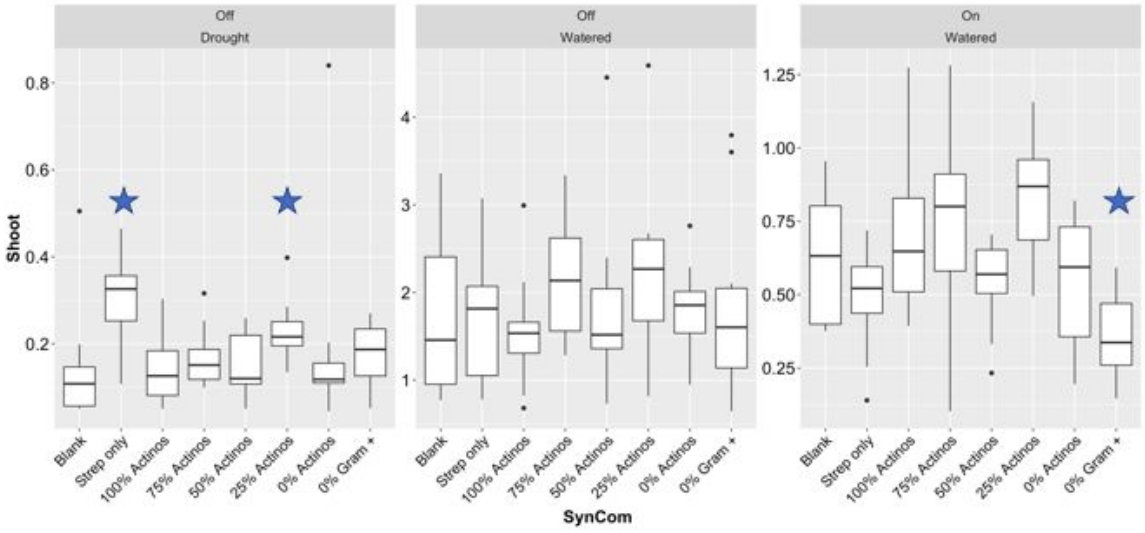

Figure 5.
Root and shoot biomass of plants from the repeated SynCom experiment. Center lines in the boxes are means, while the top and bottom lines of the boxes represent the first and third quartile. Star indicates a significant difference from the "blank" plants within the same category, as determined by a Wilcoxon test ($p<0.05$).

Table 1.
List of strains that were present in each SynCom (community name as header). The initial 3 letter/3 number code denotes the in-house strain collection identifier. Background colors indicate phyla (yellow = *Actinobacteria*, red = *Proteobacteria*, green = *Firmicutes*, blue = *Bacteroidetes*).

100% Actinos	75% Actinos	50% Actinos	25% Actinos	0% Actinos	0% Gram positives	Streptomyces only
SAI157_Streptomyces	SAI157_Streptomyces	SAI157_Streptomyces	SAI157_Streptomyces	TBS018_Pantoea	TBS018_Pantoea	SAI157_Streptomyces
SAI203_Streptomyces	SAI203_Streptomyces	SAI203_Streptomyces	SAI203_Streptomyces	TBS015_Agrobacterium	TBS015_Agrobacterium	SAI206_Streptomyces
SAI115_Promicromonospora	SAI115_Promicromonospora	SAI115_Promicromonospora	SAI115_Promicromonospora	TBS012_Agrobacterium	TBS012_Agrobacterium	SAI219_Streptomyces
SAI152_Micromonospora	SAI152_Micromonospora	SAI152_Micromonospora	SAI152_Micromonospora	TBS004_Pseudomonas	TBS004_Pseudomonas	SAI208_Streptomyces
SAI008_Kribella	SAI008_Kribella	SAI008_Kribella	TBS018_Pantoea	TBS010_Pseudomonas	TBS010_Pseudomonas	SAI055_Streptomyces
SAI079_Glycomyces	SAI079_Glycomyces	SAI079_Glycomyces	TBS015_Agrobacterium	TBS009_Agrobacterium	TBS009_Agrobacterium	
SAI206_Streptomyces	SAI206_Streptomyces	SAI206_Streptomyces	TBS012_Agrobacterium	TBS014_Agrobacterium	TBS014_Agrobacterium	
SAI095_Nocardioides	SAI095_Nocardioides	SAI095_Nocardioides	TBS004_Pseudomonas	TBS028_Pseudomonas	TBS028_Pseudomonas	
SAI105_Promicromonospora	SAI105_Promicromonospora	TBS018_Pantoea	TBS010_Pseudomonas	TBS108_Phyllobacterium	TBS108_Phyllobacterium	
SAI229_Streptomyces	SAI229_Streptomyces	TBS015_Agrobacterium	TBS009_Agrobacterium	TBS064_Methylobacterium	TBS064_Methylobacterium	
SAI225_Amycolatopsis	SAI225_Amycolatopsis	TBS012_Agrobacterium	TBS014_Agrobacterium	TBS069_Bacillus	TBS031_Pseudomonas	
SAI219_Streptomyces	SAI219_Streptomyces	TBS004_Pseudomonas	TBS028_Pseudomonas	TBS055_Bacillus	TBS063_Methylobacterium	
SAI037_Nocardioides	TBS018_Pantoea	TBS069_Bacillus	TBS069_Bacillus	TBS057_Bacillus	TBS013_Chryseobacterium	
SAI208_Streptomyces	TBS015_Agrobacterium	TBS055_Bacillus	TBS055_Bacillus	TBS013_Chryseobacterium	TBS008_Chryseobacterium	
SAI055_Streptomyces	TBS069_Bacillus	TBS013_Chryseobacterium	TBS013_Chryseobacterium	TBS008_Chryseobacterium	TBS030_Chryseobacterium	
SAI101_Amycolatopsis	TBS013_Chryseobacterium	TBS008_Chryseobacterium	TBS008_Chryseobacterium	TBS030_Chryseobacterium	TBS029_Chryseobacterium	

Chapter 4 Supplemental Figures

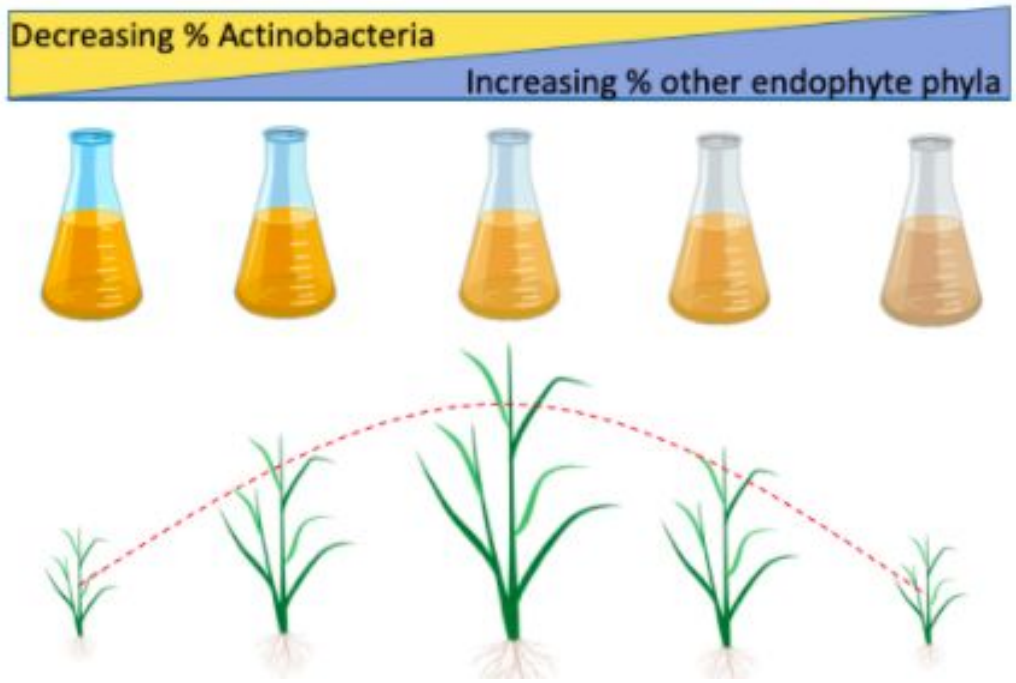

Figure S1.
Visualization of multiple SynComs with varying abundances of *Actinobacteria* and the hypothesized efficacy of growth promotion on drought stressed sorghum. Created with Biorender.

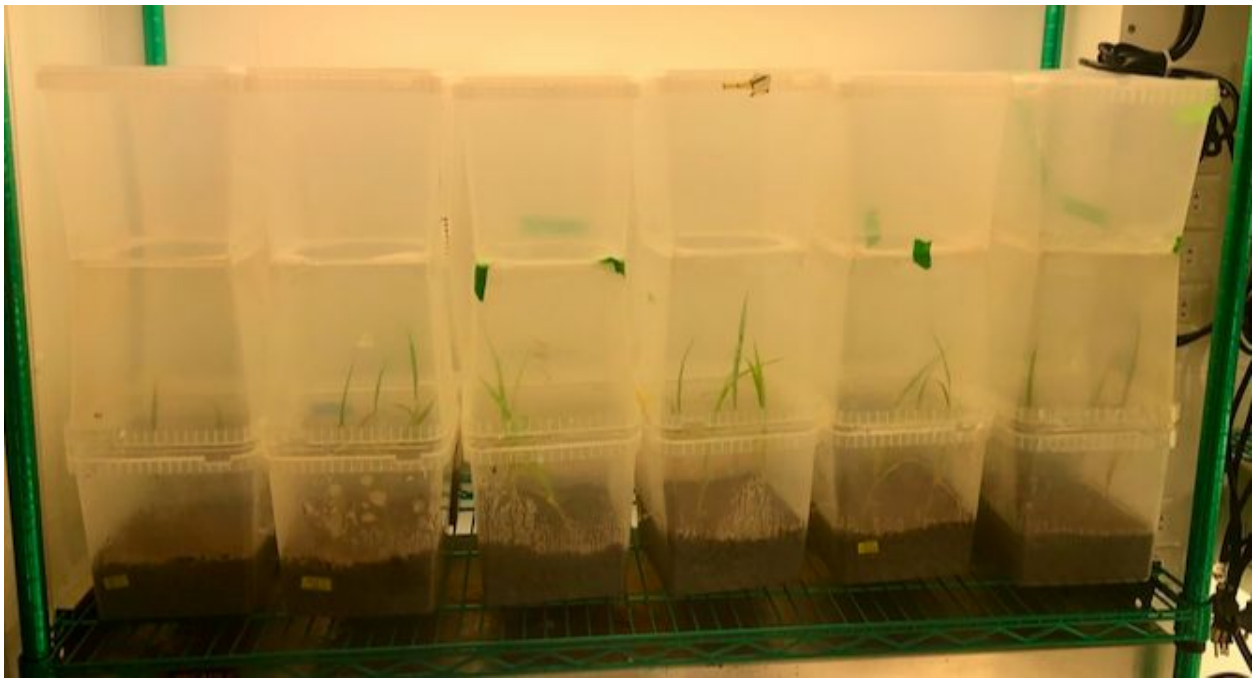

Figure S2.
Image showing modified Microbox setup to allow for additional growth space while maintaining a sterile environment.

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Appendices

A. Methods in community profiling of the root microbiome

Parts of this section have been adapted from the following with permission:
Simmons, T., Caddell, D. F., Deng, S., Coleman-Derr, D. (2018) Exploring the Root Microbiome: Extracting Bacterial Community Data from the Soil, Rhizosphere, and Root Endosphere. J. Vis. Exp. (135), e57561, doi:10.3791/57561

Introduction:
Plant-associated microbiomes consist of dynamic and complex microbial communities comprised of bacteria, archaea, viruses, fungi, and other eukaryotic microorganisms. In addition to their well-studied role in causing plant disease, plant-associated microbes can also positively influence plant health by improving tolerance to biotic and abiotic stresses, promoting nutrient availability, and enhancing plant growth through the production of phytohormones. For this reason, particular interest exists in characterizing the taxa that associate with plant root endospheres, rhizospheres, and the surrounding soil. While some microbes can be cultured in isolation on laboratory generated media, many cannot, in part because they may rely on symbiotic relationships with other microbes, grow very slowly, or require conditions that cannot be replicated in a lab environment. Because it circumvents the need for the cultivation and is relatively inexpensive and high-throughput, sequence-based phylogenetic profiling of environmental and host-associated microbial samples has become a preferred method for assaying microbial community composition.

The selection of appropriate sequencing technologies provided by various Next Generation Sequencing (NGS) platforms (Goodwin, McPherson, and McCombie 2016) is dependent on the users' needs, with important factors including: desired coverage, amplicon length, expected community diversity, as well as sequencing error-rate, read-length, and the cost-per-run/megabase. Another variable that needs to be considered in amplicon-based sequencing experiments is what gene will be amplified and what primers will be used. When designing or choosing primers, researchers are often forced to make tradeoffs between the universality of amplification and the taxonomic resolution achievable from the resulting amplicons. For this reason, these types of studies often chose primers and markers that selectively target specific subsets of the microbiome. Evaluating the composition of bacterial communities is commonly accomplished by sequencing one or more of the hypervariable regions of the bacterial 16S rRNA gene (Soergel et al. 2012; Takahashi et al. 2014). In this study, we describe an amplicon based sequencing protocol developed for a NGS platform that targets the 500 bp V3-V4 region of the bacterial 16S rRNA gene, which allows for broad amplification of bacterial taxa while also providing sufficient variability to distinguish between different taxa. Additionally, this protocol can easily be adapted for use with other primer sets, such as those targeting the ITS2 marker of fungi or the 18S rRNA subunit of eukaryotes.

While other approaches such as shotgun metagenomics, metatranscriptomics and single-cell sequencing, offer other advantages including resolved microbial genomes and more direct

measurement of community function, these techniques are typically more expensive and computationally intensive than the phylogenetic profiling described here (Poretsky et al. 2014). Additionally, performing shotgun metagenomics and metatranscriptomics on root samples yields a large percentage of reads belonging to the host plant genome, and methods to overcome this limitation are still being developed (Sharpton 2014; Jiao et al. 2006).

As with any experimental platform, amplicon-based profiling can introduce a number of potential biases which should be considered during the experimental design and data analysis. These include the methods of sample collection, DNA extraction, selection of PCR primers, and how library preparation is performed. The choices made for each of the above can significantly impact the amount of usable data generated, and can also hinder the efforts to compare results between studies. For example, the method of removing rhizosphere bacteria(Richter-Heitmann et al. 2016) and the use of different extraction techniques or choice of DNA extraction kits (Mahmoudi et al. 2011; Vishnivetskaya et al. 2014) have been shown to significantly impact downstream analysis, which leads to different conclusions regarding which microbes are present and their relative abundances. As a result of the large degree to which amplicon-based profiling can be customized, making comparisons across studies can be challenging. The Earth Microbiome Project has suggested that researchers studying complex systems such as the plant-associated microbiome would benefit from the development of standardized protocols as a means of minimizing the variability caused by the application of different methods between studies (Busby et al. 2017; Thompson et al. 2017). Here, we discuss many of the above topics and offer suggestions as to best practices where appropriate.

The protocol demonstrates the process of collecting soil, rhizosphere, and root samples from *Sorghum bicolor* and extracting DNA using a well-established DNA isolation kit (Thompson et al. 2017). Additionally, our protocol includes a detailed amplicon sequencing workflow, using a commonly utilized NGS platform, to determine the structure of the bacterial communities (Caporaso et al. 2012; Kozich et al. 2013; Degnan and Ochman 2012). This protocol has been validated for the use in a wide range of plant hosts in a recent published study of the roots, rhizosphere, and associated-soils of 18 monocot species including *Sorghum bicolor, Zea mays,* and *Triticum aestivum* (Naylor et al. 2017). This method has also been validated for use with other marker genes, as demonstrated by its successful application to studying the fungal ITS2 marker gene in studies of the agave microbiome (Desgarennes et al. 2014; Coleman-Derr et al. 2016) and strawberry microbiome (De Tender et al. 2016).

Protocol:

1. Collection and Separation of Root Endosphere, Rhizosphere, and Soil Samples

1.1. Prior to entering the field, autoclave ultrapure water (at least 90 mL of water per sample) to sterilize. Prepare epiphyte removal buffer (at least 25 mL per sample) by adding 6.75 g of KH_2PO_4, 8.75 g of K_2HPO_4, and 1 mL of Triton X-100, to 1 L of sterile water. Sterilize the buffer using a vacuum filter with 0.2 μm pore size.

1.1.1. For steps 1.2 to 1.5, wear clean gloves sterilized with ethanol at all times and replace the gloves between each sample to prevent contamination. Sterilize all equipment with 70% ethanol and wipe clean all equipment between samples. Before sampling, determine the optimal sampling depth for your experiment, and be consistent with all soil and root collections.

1.2. To collect bulk soil samples, use an ethanol-sterilized soil core collector to obtain soil that is free of plant roots by collecting a core approximately 23 to 30 cm from the base of the plant.

1.3. Transfer the soil to a plastic bag, homogenize the soil by gentle shaking, and transfer an aliquot of the soil sample to fill one 2 mL tube (approximately 600 mg). Immediately place the 2 mL tube on dry ice or flash freeze the tube in liquid N_2 until ready to proceed with DNA extraction (step 2).

1.3.1. In some environments, the surrounding soil can contain plant material. In this case, use a sterilized 2 mm sieve to separate the plant debris from the soil prior to placing in the plastic bag.

1.4. To collect the root and rhizosphere, use an ethanol-sterilized shovel to dig up the plant, taking care to obtain as much of the root biomass as possible. Depth is dependent upon the plant; while small plants such as wheat can be removed by digging several cm, larger plants such as sorghum may require 30 cm or more. Gently shake off excess soil from the roots until there is approximately 2 mm of soil adhering to the root surface.

Note: Take care when working with small plants, with fragile roots, or in dry, high-clay content soils. Ideally, there should only be a thin layer of soil remaining on the roots after shaking. If large aggregates of soil remain, a rubber mallet can be used to dislodge the soil by gently hitting the base of the shoot. If the amount of soil remaining after this process exceeds or falls short of 2 mm, the approximate thickness should be noted.

1.5. For large plants, use sterile scissors and/or shears to cut a representative subsection of roots and place a minimum of 500 mg of root tissue into a 50 mL conical vial. For smaller grasses, place the entire root system into the vial. Add enough epiphyte removal buffer to cover the roots, then immediately place the sample on dry ice or flash freeze the sample in liquid N_2.

Note: Take care not to overfill the 50 mL conical vial, as it will make washing the roots difficult; there should be enough empty space such that the epiphyte buffer is able to flow to the bottom, surround the roots throughout the vial, and cover the top. Because some grasses have more root biomass than will fit into a 50 mL conical vial, a subsection of the roots should be collected. However, it should be noted that cutting the roots could lead to endophytic bacteria being washed out into the rhizosphere fraction, so breaking roots should be minimized. If samples are not processed immediately after returning to lab, they can be stored at -80 °C.

1.6. To separate the rhizosphere from the roots, thaw the root sample on ice, then sonicate the root samples at 4 °C for 10 min with pulses of 160 W for 30 s, separated by 30 s. Transfer the

roots into a chilled (4 °C), clean 50 mL tube using sterile forceps. Do not dispose the original tube with buffer and soil; this is the rhizosphere fraction (Figure 1).

1.7. Centrifuge the tube containing buffer and rhizosphere for 10 mins at 4 °C, 4,000 x g. Decant the supernatant, flash freeze the tube containing the rhizosphere fraction in liquid N_2, and store the rhizosphere fraction at -80 °C until ready to proceed with DNA extraction (step 2).

1.8. To wash the roots, add approximately 20 mL of chilled (4 °C) sterile water to the root fraction. Wash the root by shaking vigorously (by hand or mixer, for 15-30 s), and then drain the water.

1.9. Repeat this step at least twice, until no soil remains on the root surface. If the DNA extraction (step 2) is not performed immediately, wrap the roots in sterile aluminum foil, flash freeze the roots in liquid N_2, and store the samples at -80 °C until ready to proceed with DNA extraction.

2. DNA Extraction

Note: Throughout steps 2 and 3, clean gloves sterilized with ethanol should be worn at all times and all work should be performed on a surface sterilized with ethanol.

2.1. Extract DNA from the soil and rhizosphere samples.

2.1.1. Use a sterile spatula to quickly transfer 250 mg of soil and rhizosphere from steps 1.3 and 1.7 into separate collection tubes provided in a commercial DNA isolation kit designed for extraction from soil, then proceed with DNA isolation using the kit supplier's protocol.

2.1.2. After eluting the DNA in the elution buffer supplied by the DNA isolation kit, store the DNA at -20 °C until ready to proceed with step 3.

2.2. Extract DNA from the root samples.

2.2.1. Chill a sterilized mortar and pestle using liquid N_2. Measure out 600 to 700 mg of root tissue and place the tissue into the mortar. Carefully, add enough liquid N_2 to cover the roots.

2.2.2. Grind the roots into small pieces. Continue the process of adding liquid N_2 and grinding (at least two times, be consistent between samples), until the roots are a fine powder. Ensure that the root tissue does not thaw during this step.

CAUTION: Use appropriate personal protective equipment (lab coat, protective eyewear, and cryogenic gloves) when working with liquid N_2.

Note: In the event of a low-quality DNA extraction, it can be beneficial to grind excess roots into a powder and store the powder at -80 °C.

2.2.3. Quickly, before the root powder begins to thaw, use a sterile spatula to transfer the root powder into pre-weighed 1.5 mL tubes on ice. Record the weight of the tube and powder. Typically, 300-400 mg of powder is transferred.

2.2.4. Use a sterile spatula to quickly transfer 150 mg of root powder to the collection tube provided in a commercial DNA isolation kit designed for extraction from soil, then proceed with DNA isolation using the kit supplier's protocol.

Note: For some root samples, there can be a high concentration of organics remaining in the DNA pellet, which prevents the amplification of the DNA during PCR, especially when a different DNA extraction protocol (e.g., CTAB extraction) is used. If necessary, clean the DNA by following the instructions provided in the environmental DNA clean-up kit.

2.3. Measure the concentration of all DNA samples using a high-sensitivity benchtop fluorometer.

2.3.1. Add 1-20 µL of each eluted DNA sample into tubes provided in the dsDNA high-sensitivity assay kit. Add fluorometer working solution (1:200 dye:buffer) up to 200 µL.

2.3.2. Prepare two additional tubes containing 10 µL of DNA standard 1 (0 ng/µL DNA) or 10 µL of standard 2 (100 ng/µL), and add 190 µL of fluorometer working solution to each standard.

2.3.3. Measure the concentration of the standards and each sample. If it is not done automatically, calculate the DNA concentration from the absorbance output by a linear regression of the two standards.

3. Amplicon Library Preparation and Submission

3.1. Set up materials for the amplification reaction.

3.1.1. Thaw DNA samples at 4 °C and keep them on ice throughout step 3. Randomize the order of DNA samples to minimize bias due to the location on the PCR plate (Table 2.)

3.1.2. In a 96-well PCR plate, dilute DNA from each sample in molecular-grade water to 5 ng/µL in a total volume of 20 µL. Add 20 µL of molecular-grade water to the four corner wells as negative controls for amplification (blanks) (Table 2).

3.1.3. Arrange the barcoded primers (10 µM) in either PCR strip tubes or a 96-well plate such that they can be added with a multi-channel pipette (Figure 2).

3.1.4. Prepare sufficient PCR master mix to amplify each DNA sample in triplicate. Triplicate volumes per sample are as follows: 1.5 µL of BSA (20 mg/mL), 30 µL of pre-made master mix (composed of PCR buffer, $MgCl_2$, dNTPs, and Taq DNA polymerase), 0.57 µL of chloroplast PNA (100 µM), 0.57 µL of mitochondrial PNA (100 µM), and 33.36 µL of molecular grade water.

3.1.5. Pour the master mix into a sterile 25 mL of multichannel pipette reservoir and distribute 66 µL of master mix into each well of a new 96-well PCR plate using a multichannel pipette.

Note: When calculating reagent volumes for the master mix, make sure to also include the 4 blank wells per plate.

3.1.6. Using a multi-channel pipette, add 6 µL of 5 ng/µL DNA (from the normalized DNA plate) to the master mix plate. Then add to the master plate 1.5 µL of 10 µM forward primer such that each column has a different forward barcode, and 1.5 µL of 10 µM reverse primer such that each row has a different reverse barcode (Figure 2).

Note: Prior to adding primers, the randomized plates and master mix could be used to amplify the ITS or ITS2 fungal genes if different primers were added. If this is the case, a similar primer design can be used.

3.1.7. Spin down the plate briefly at 3,000 x g. Use a multi-channel pipette to mix gently, then divide into three plates with 25 µL of reaction volumes.

Note: Although three replicates are not strictly necessary, it decreases the impact of technical variability.

3.2. Amplify the DNA in each plate using a thermocycler set to the following conditions: 180 s at 98 °C, 30 cycles of: 98 °C for 45 s (denaturing), 78 °C for 10 s (PNA annealing), 55 °C for 60 s (primer annealing), and 72 °C for 90 s (extension), then 600 s at 72 °C followed by a 4 °C hold step. After the amplification, pool the three replicate plates into one single 96-well plate.

3.3. Quantify the DNA using high-sensitivity fluorometer reagents in a 96-well plate reader.

3.3.1. Add 2 µL of each PCR product to a 96-well microplate, along with 98 µL of fluorometer working solution (1:200 dye:buffer). Include 4 wells as standards: 5 µL of DNA standard 1 (0 ng/µL DNA), 1 µL of standard 2 (10 ng/µL), 2 µL of standard 2 (20 ng/µL), and 5 µL of standard 2 (50 ng/µL). Then add fluorometer working solution for a final volume of 100 µL.

Note: Each sample can be measured using a benchtop fluorometer as described in step 2.3 if a plate reader is not available.

3.3.2. Calculate the DNA concentration from the absorbance output by a linear regression of the four standards.

3.3.3. For the successfully amplified barcoded products (those that have a concentration greater than 15 ng/µL), pool 100 ng of each sample into a single 1.5 mL tube (Table 2).

3.3.4. Calculate the average volume of samples added to the pool by using the =AVERAGE() function in a spreadsheet program. Add that volume of the "blank" PCR products to the pooled samples.

Note: Since the "blank" PCR products have their own unique barcode combinations, they can be sequenced to check for any laboratory contaminants.

3.4. Measure the concentration of the pooled product using a benchtop fluorometer as described in step 2.3, and take 600 ng of DNA and dilute in molecular-grade water to a final volume of 100 µL in a 1.5 mL tube. Store the remaining pooled product at -20 °C.

3.5. Wash the 600 ng DNA aliquot by following the established PCR purification process for use with paramagnetic purification beads in a 96-well format (per manufacturer instructions) with a few exceptions.

3.5.1. Make a fresh 600 µL aliquot of 70% ethanol. Shake the bottle of magnetic beads to re-suspend beads that settle to the bottom.

3.5.2. Add 1x volume (100 µL) of bead solution to the 600 ng aliquot of DNA. Mix thoroughly by pipetting 10 times. Incubate for 5 min at room temperature.

3.5.3. Place the tube onto the magnetic stand for 2 min (or until solution is clear) to separate beads from solution. While the tube is still in the magnetic stand, aspirate the clear supernatant carefully without touching the magnetic beads, and discard the clear supernatant.

Note: At this point, the amplicon products are bound to the magnetic beads. Any beads that are disturbed or lost during aspiration will result in a loss of DNA.

3.5.4. Leave the tube in the magnetic stand and add 300 µL of 70% ethanol to the tube; incubate at room temperature for 30 s. Aspirate out the ethanol and discard. Repeat this process, and remove all ethanol after the second wash. Remove the tube from the magnetic stand, and air dry for 5 min.

3.5.5. Add 30 µL of molecular-grade water to the dried beads and mix by pipetting 10 times. Incubate at room temperature for 2 min. Return the tube to the magnetic stand for 1 min to separate the beads from solution. Transfer the eluate to a new tube.

Note: Magnetic beads will not affect downstream reactions.

3.6. Measure the final concentration of cleaned, pooled DNA using a benchtop fluorometer as described in step 2.3, and dilute an aliquot to 10 nM in a final volume of 30 µL, or to the concentration and volume preferred by the sequencing facility.

3.7. Utilize the services of a sequencing facility to sequence the DNA on a NGS platform, 2 x 300 bp paired-end sequencing.

Representative Results:
Performing the recommended protocol should result in a dataset of indexed paired-end reads that can be matched back to each sample and assigned to either a bacterial OTU or ESV (Exact Sequence Variant; also referred to as ASV - amplicon sequence variant and sOTU - sub-operational taxonomic unit), depending on downstream analysis. In order to obtain high-quality sequence data, care must be taken at each step to maintain consistency between samples and minimize the introduction of any potential bias during sample processing or library preparation. After collecting, processing, and extracting DNA from samples (steps 1 and 2), the resulting eluate should appear clear and free of organics that would inhibit amplification. While purity can be verified by measuring each DNA sample via a microvolume spectrophotometer, we have found that the soil DNA extraction kit reliably removes all contaminants. As a result of the predictable DNA quality, quantification methods that rely on fluorescence-based dyes that specifically bind DNA are more appropriate than those based on UV absorbance (Kapp et al. 2015; Simbolo et al. 2013; O'Neill et al. 2011). Prior to PCR amplification, soil and rhizosphere samples average around 10 ng/µL DNA, while root samples typically have a mean concentration of approximately 30 ng/µL (Table 2).

Following amplification of the environmental DNA (step 3), success or failure can be determined by measuring the concentration of the PCR product via benchtop fluorometer reagents on a plate reader, if available, or manually (Table 2). In our experience, successful amplifications that result in high-quality amplicon data yield greater than 15 ng/µL PCR products. If there are multiple failures on a plate, the positional arrangement within the plant and sample type of failed samples may help determine the problem. For instance, if they are all adjacent on the plate it may indicate pipette error, whereas if they are all in the same row or column, it could suggest issues with a specific primer. If they all belong to the same sample type, it might suggest problems with sample processing or DNA extraction.

It is important to check the compatibility of the universal PNAs with your specific plant system bioinformatically during experimental design in order to verify that they will block amplification of chloroplast and mitochondrial 16S genes. Following the amplification step, it is not apparent whether the PNAs successfully bound to mitochondrial and chloroplast templates; this is only revealed after sequencing (Figure 3). To help ensure that the PNAs will effectively block contaminant amplification, an alignment of the PNA sequence to each chloroplast and mitochondrial 16S rRNA gene (there may be multiple copies) for the plant host being investigated should not reveal any mismatches. Even a single mismatch to the 13 bp PNA sequence, especially in the middle of the PNA clamp, can drastically reduce the effectiveness, as in the case of the provided chloroplast PNA sequence and the chloroplast 16S rRNA gene of Lactuca sativa (lettuce) (Figure 3).

Since an equal amount of amplified DNA is pooled per sample, there should be an approximately even number of reads obtained per sample after sequencing and sorting reads based on their barcoded index (Figure 4). The majority of these reads should match to bacterial taxa; any eukaryotic, mitochondrial, or chloroplast matches should be discarded. Note also that depending on the analysis pipeline and taxonomic database chosen, chloroplast and mitochondrial reads can

mistakenly be classified as belonging to bacterial lineages, often Cyanobacteria and Rickesttia, respectively (Figure 3). A degree of manual curation is often prudent to check for these common mis-assignments. Specific details will depend on the choice of analysis, but relative abundance profiles should generally be similar (no significant difference) among biological replicates and significantly different between soil, rhizosphere, and root samples (Figure 5). It is important to note, however, that while there may be no significant difference between biological replicates, it is important to collect at least three replicates per sample in order to verify that this is the case.

Methods for interpreting the data obtained in these experiments are hotly debated amongst microbial ecologists. Until recently, amplicon sequence analysis has been dependent upon grouping reads into OTUs (Operational Taxonomic Units). However, these are problematic because: 1) they are based on a somewhat arbitrary threshold of 97% similarity, 2) diversity is often underestimated, and 3) there can be low taxonomic resolution. Recently developed tools such as DADA2, Deblur, and UNOISE2(Callahan et al. 2016b; Amir et al. 2017; Edgar 2016) are able to sort reads into ESVs (Exact Sequence Variants), which solves some problems presented when using OTUs. Caveats to using ESVs include: 1) artificial increases in diversity due to differences in rRNA copies within a species, and 2) increased sensitivity to PCR and sequencing errors(Nguyen et al. 2016; Callahan, McMurdie, and Holmes 2017).

Discussion:
This protocol demonstrates an established pipeline for exploring root endosphere, rhizosphere, and soil microbial community compositions, from field sampling to sample processing and downstream sequencing. Studying root-associated microbiomes presents unique challenges, due in part to the inherent difficulties in sampling from soil. Soils are highly variable in terms of physical and chemical properties, and different soil conditions can be separated by as little as a few millimeters (O'Brien et al. 2016; Fierer and Lennon 2011). This can lead to samples which are collected from adjacent sampling sites having considerably different microbial community compositions and activitie s(Fierer 2017; Buckley and Schmidt 2003). Thus, using soil core collectors and shovels to maintain consistent sampling depths and homogenization prior to DNA extraction are essential to reproducibility within root microbiome studies. It is also essential to efficiently separate the rhizosphere and root fractions; using a harsh method of root surface sterilization can potentially lyse endophytes within roots prior to DNA extraction, while a more conservative wash may not remove all microbes from the root surface (Richter-Heitmann et al. 2016). Another key factor that can negatively impact or disrupt sequencing results is bacterial contamination, which can come from many sources and is sometimes impossible to distinguish from the sampled environmental bacteria (Salter et al. 2014; Weiss et al. 2014). For this reason, careful sterilization of sampling tools, experimental materials, and working environments are vital in order to avoid contamination.

After sampling, obtaining high quality DNA is a high priority for successful downstream analyses. In our experience, DNA extraction from field grown root samples through alternative methods, such as through CTAB-based extraction, often contain substantially greater quantities of humic acids and other compounds compared to rhizosphere and soil samples; these compounds can prevent the enzymatic activity of the DNA polymerase during PCR amplification, even at low concentrations (Sutlović et al. 2005; Sutlovic et al. 2008). Using DNA

extraction kits designed for soils on root samples, as opposed to a CTAB extraction followed by a phenol chloroform clean-up, can effectively rid samples of humic acids and will result in high quality DNA (Aleklett et al. 2015; Bogas et al. 2015; Hiscox et al. 2015; Zhang and Yao 2015). Accordingly, we recommend using a commercially available DNA extraction kit for root samples as well. It should be noted that the goal is to obtain microbial genomic DNA from plant roots. Thus, thorough and consistent root grinding is important to break down the plant tissue and lyse the microbial cells to release microbial DNA without introducing bias between samples due to variation in grinding pressure and time.

Following careful extraction of DNA from samples, there are two main sources for problems during amplification: 1) contamination of plant tissues with plant endosymbionts (chloroplast and mitochondria) and 2) selection of 16S rRNA region to amplify. The amplification from chloroplast or mitochondria 16S rRNA sequences can generate >80% of the sequences in root samples (Ghyselinck et al. 2013), and more in leaf tissues, though the amount of contamination is dependent on the choice of primers. Thus, PNA clamps are necessary during the PCR step to suppress plant host chloroplast and mitochondrial 16S contamination (von Wintzingerode et al. 2000; Lundberg et al. 2013). However, different plant species can have variation in the chloroplast and mitochondrial 16S sequence (Lundberg et al. 2013); therefore, it is essential to confirm the sequence of the chloroplast and mitochondrial 16S genes of the plant being studied prior to library sequencing, in order to determine if alternate PNA oligos are needed (Figure 3). Additionally, the 16S rRNA gene consists of nine hypervariable regions flanked by nine conservative regions; different results can be obtained from the same community depending upon which hypervariable region is amplified (Cruaud et al. 2014). Previous studies have found the V4 region to be one of the most reliable for assigning taxonomy (B. Yang, Wang, and Qian 2016) and it has been used for other extensive microbiome surveys (Thompson et al. 2017). Lengthening the target to the V3-V4 region is suggested here to increase variability and improve taxonomic resolution.

In this protocol, we demonstrated a pipeline to perform 16S rRNA amplicon sequencing via Next Generation Sequencing (NGS) for studying microbial community compositions of environmental samples (Caporaso et al. 2012). We recommend using amplicon sequencing as a tool for phylogenetic profiling because it is relatively inexpensive, high-throughput and does not require extensive computational expertise or resources to analyze. While our method focuses on analyzing the bacterial fraction of the microbiome, it can easily be adapted to investigate fungi. The protocol is identical through step 2, and the only difference in step 3 is what primers would be used during amplification. However, it is worth nothing that amplicon based profiling is not without limitations. By sequencing a single marker gene, no information is obtained regarding the functional capacity of the community. Additionally, the taxonomic resolution can be quite low, especially when sequencing from environments with a high percentage of uncharacterized microbes. However, sequencing technologies are rapidly evolving, and we anticipate the potential to deal with some of these shortcomings by adapting this protocol for use with other sequencing platforms. Finally, as mentioned in the introduction, shotgun metagenomics and metatranscriptomics can easily be performed on soil and rhizosphere samples, and methods to eliminate plant contamination from plant tissues are currently being explored. Experimental designs which pair amplicon-based approaches and other metagenomic techniques can be

particularly effective in complex communities where high species diversity and uneven representation of taxa can prevent shotgun data from accurately characterizing the less dominant members.

Figures:

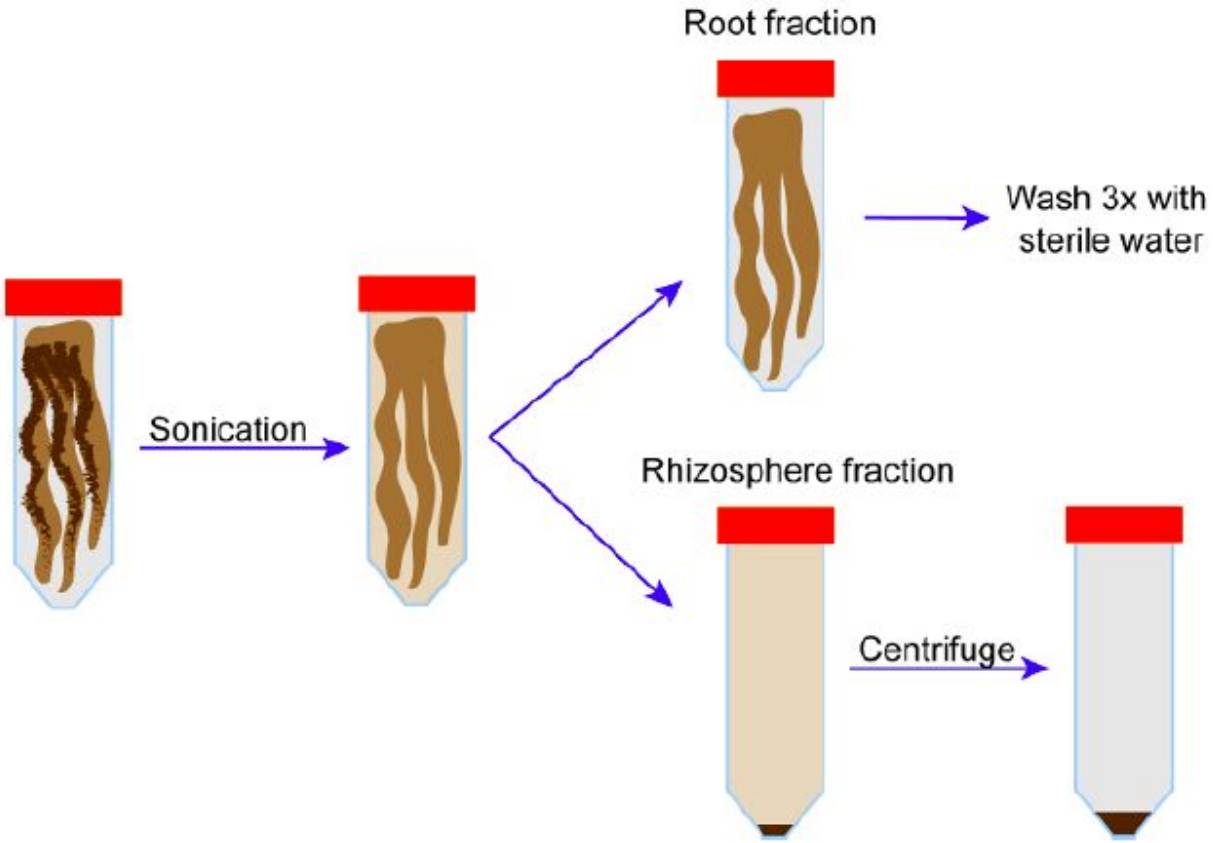

Figure 1.
Separation of root and rhizosphere fractions. Flowchart displaying the steps for separating the rhizosphere from the root samples, followed by washing the roots with sterile water to remove any remaining rhizoplane organisms.

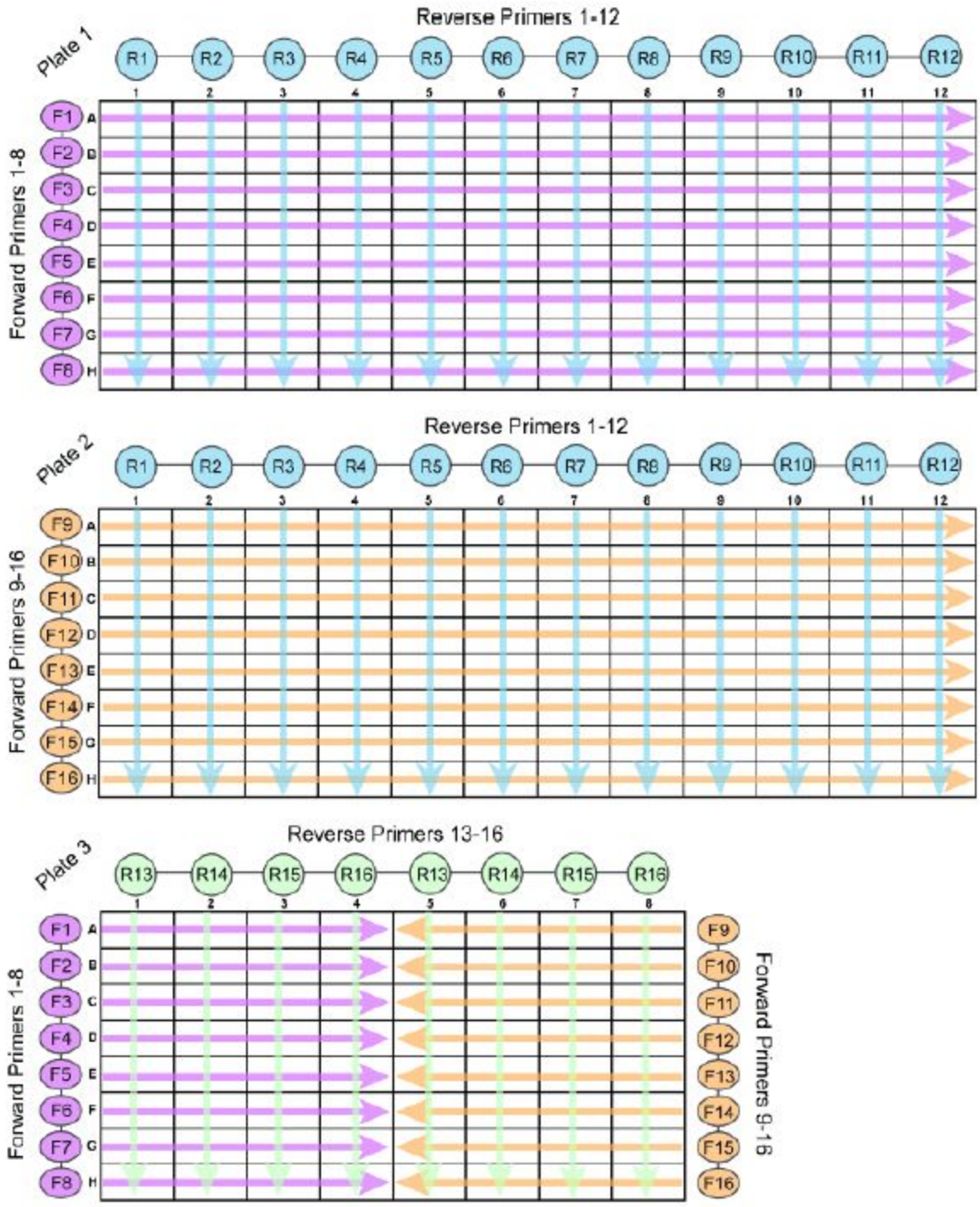

Figure 2.
Example of stock primer layout for amplification and distribution within plates. Stock primers (Table 1) can be prepared in strip tubes for optimal distribution within 96-well plates (each strip of primers is represented by a different color; purple for forward primers 1 - 8, orange for forward primers 9 - 16, blue for reverse primers 1 - 12, and green for reverse primers 13 - 16.) In this case, 16 forward and 16 reverse primers can be distributed efficiently with a multi-channel pipette such that each well has a unique barcode combination.

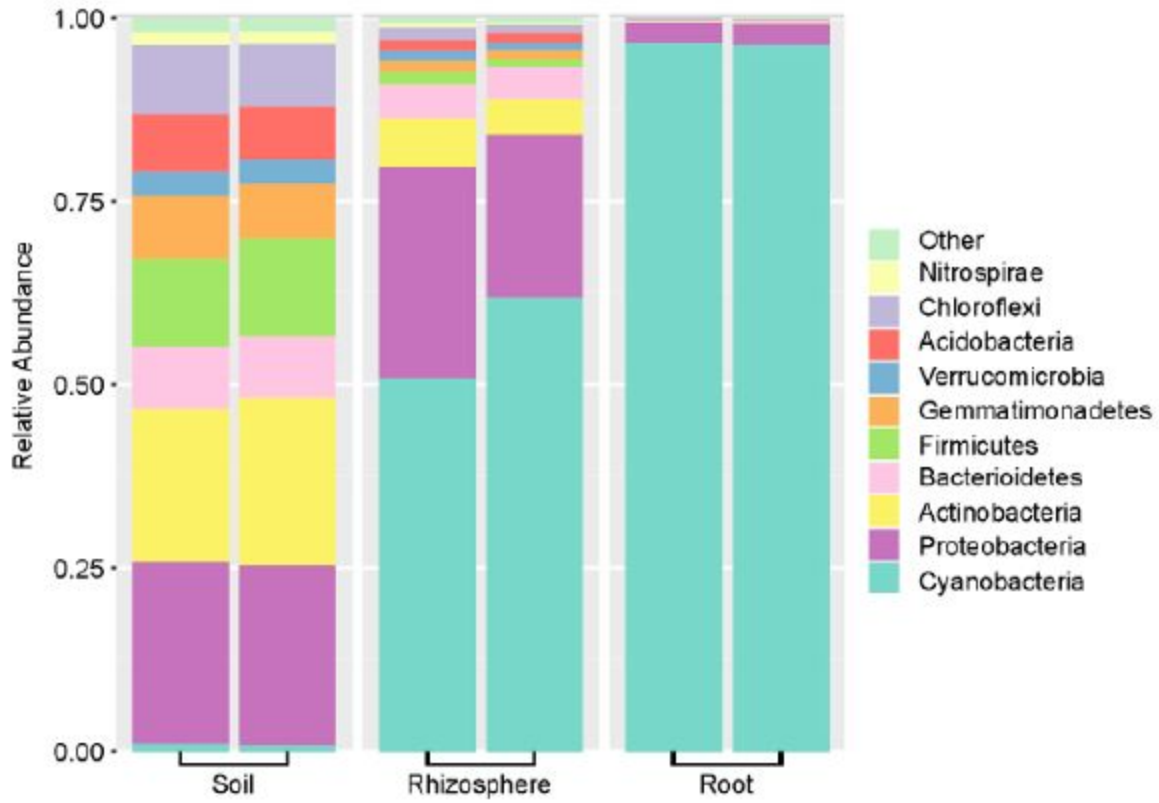

Figure 3.
Results that suggest chloroplast PNA is ineffective. Representative result from rhizosphere ("Rhizo"), root, and soil samples from lettuce that were non-treated (NT) or treated (VT) with a biological soil amendment. The PNA sequence used to block chloroplast contamination of most plants is GGCTCAACCCTGGACAG27. However, lettuce contains a mismatch in the chloroplast 16S ribosomal RNA gene (GGCTCAACTCTGGACAG). This renders the PNA ineffective, resulting in a high relative abundance of reads that match to Cyanobacteria in rhizosphere and root samples.

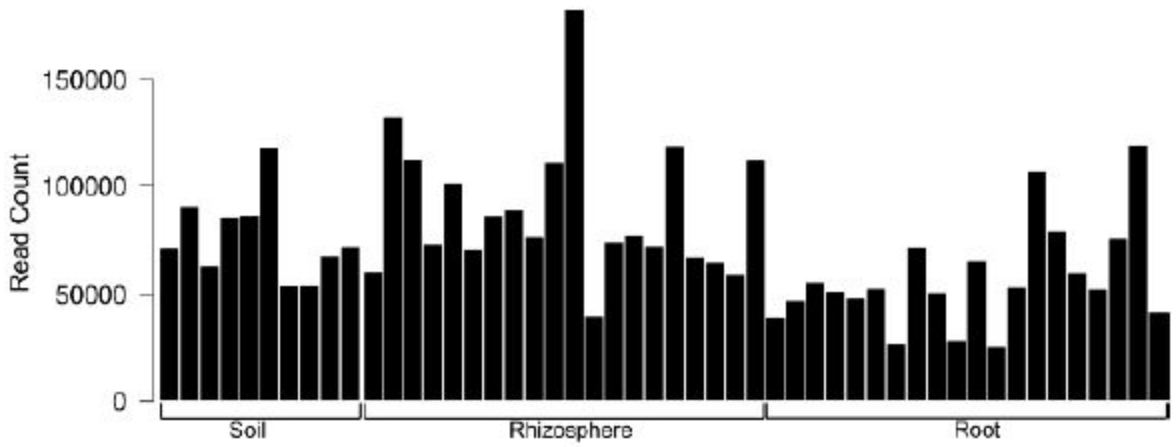

Figure 4.
Distribution of read counts among samples in a library. Bar chart showing number of read counts (y-axis) from different samples (bars, x-axis), matched by the barcode combination in the read. The number of reads per sample can vary based on how many samples are in the library; this subset was sequenced in a library of 192 samples.

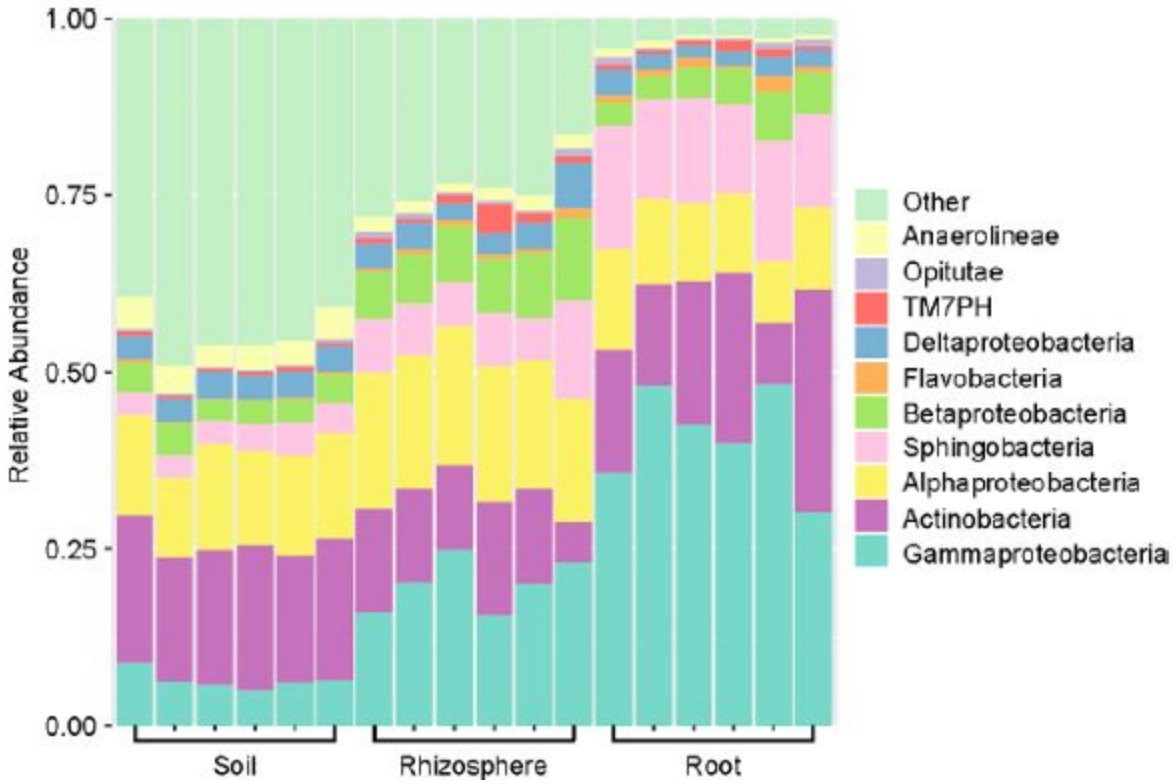

Figure 5.
Relative abundance of the top 12 classes in root, rhizosphere, and soil communities. Stacked bar chart showing relative abundance of classes present in a representative 16S dataset containing 6 replicates for each sample type (bulk soil, rhizosphere, and root endosphere).

Table 1. Primers for amplifying the V3-V4 region of the 16S rRNA gene. Primers are composed of, sequentially: an adapter for a common NGS platform, a unique barcode, the primer for NGS, a spacer region of variable length to shift the frame for sequencing, and a universal PCR primer that amplifies either 341F or 785R of the 16S rRNA gene. The number of primers needed is dependent upon how many samples are sequenced per library; a combination of 16 forward and 16 reverse primers is sufficient for 244 samples (256 primer combinations with 12 used for blank wells during PCR (Figure 2)).

Primer Set Name	Primer Name	Primer short name	PRIMER SEQUENCE				
			NGS Adapter	Barcode	NGS Sequencing Primer	SPACER	16S rRNA PRIMER
Standard Forward Primer 1-16	341F.1	F1	AATGATACGGCGACCACCGAGATCTACAC	CCTAAACTACGG	TCTTTCCCTACACGACGCTCTTCCGATCT		CCTACGGGNBGCASCAG
	341F.2	F2	AATGATACGGCGACCACCGAGATCTACAC	GTGGTATGGGAG	TCTTTCCCTACACGACGCTCTTCCGATCT	T	CCTACGGGNBGCASCAG
	341F.3	F3	AATGATACGGCGACCACCGAGATCTACAC	TGTTGCGTTTCT	TCTTTCCCTACACGACGCTCTTCCGATCT	GT	CCTACGGGNBGCASCAG
	341F.4	F4	AATGATACGGCGACCACCGAGATCTACAC	ACAGCCACCCAT	TCTTTCCCTACACGACGCTCTTCCGATCT	CGA	CCTACGGGNBGCASCAG
	341F.5	F5	AATGATACGGCGACCACCGAGATCTACAC	GTTACGTGGTTG	TCTTTCCCTACACGACGCTCTTCCGATCT	ATGA	CCTACGGGNBGCASCAG
	341F.6	F6	AATGATACGGCGACCACCGAGATCTACAC	TACCGGCTTGCA	TCTTTCCCTACACGACGCTCTTCCGATCT	TGCGA	CCTACGGGNBGCASCAG
	341F.7	F7	AATGATACGGCGACCACCGAGATCTACAC	CACCTTACCTTA	TCTTTCCCTACACGACGCTCTTCCGATCT	GAGTGG	CCTACGGGNBGCASCAG
	341F.8	F8	AATGATACGGCGACCACCGAGATCTACAC	TTAACTGGAAGC	TCTTTCCCTACACGACGCTCTTCCGATCT	CCTGGAG	CCTACGGGNBGCASCAG
	341F.9	F9	AATGATACGGCGACCACCGAGATCTACAC	TACCGCCTCGGA	TCTTTCCCTACACGACGCTCTTCCGATCT		CCTACGGGNBGCASCAG
	341F.10	F10	AATGATACGGCGACCACCGAGATCTACAC	ACTTTAAGGGTG	TCTTTCCCTACACGACGCTCTTCCGATCT	T	CCTACGGGNBGCASCAG
	341F.11	F11	AATGATACGGCGACCACCGAGATCTACAC	CCATCACATAGG	TCTTTCCCTACACGACGCTCTTCCGATCT	GT	CCTACGGGNBGCASCAG
	341F.12	F12	AATGATACGGCGACCACCGAGATCTACAC	GAGCAACATCCT	TCTTTCCCTACACGACGCTCTTCCGATCT	CGA	CCTACGGGNBGCASCAG
	341F.13	F13	AATGATACGGCGACCACCGAGATCTACAC	ATGTCCGACCAA	TCTTTCCCTACACGACGCTCTTCCGATCT	ATGA	CCTACGGGNBGCASCAG
	341F.14	F14	AATGATACGGCGACCACCGAGATCTACAC	TGTCTCGCAAGC	TCTTTCCCTACACGACGCTCTTCCGATCT	TGCGA	CCTACGGGNBGCASCAG
	341F.15	F15	AATGATACGGCGACCACCGAGATCTACAC	CGCGGTTACTAA	TCTTTCCCTACACGACGCTCTTCCGATCT	GAGTGG	CCTACGGGNBGCASCAG
	341F.16	F16	AATGATACGGCGACCACCGAGATCTACAC	GAGACTATATGC	TCTTTCCCTACACGACGCTCTTCCGATCT	CCTGGAG	CCTACGGGNBGCASCAG
Standard Reverse Primer 1-16	785R.1	R1	CAAGCAGAAGACGGCATACGAGAT	CCTAAACTACGG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		GACTACNVGGGTATCTAATCC
	785R.2	R2	CAAGCAGAAGACGGCATACGAGAT	GTGGTATGGGAG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	A	GACTACNVGGGTATCTAATCC
	785R.3	R3	CAAGCAGAAGACGGCATACGAGAT	TGTTGCGTTTCT	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TC	GACTACNVGGGTATCTAATCC
	785R.4	R4	CAAGCAGAAGACGGCATACGAGAT	ACAGCCACCCAT	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	CTA	GACTACNVGGGTATCTAATCC
	785R.5	R5	CAAGCAGAAGACGGCATACGAGAT	GTTACGTGGTTG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GATA	GACTACNVGGGTATCTAATCC
	785R.6	R6	CAAGCAGAAGACGGCATACGAGAT	TACCGGCTTGCA	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ACTCA	GACTACNVGGGTATCTAATCC
	785R.7	R7	CAAGCAGAAGACGGCATACGAGAT	CACCTTACCTTA	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TTCTCT	GACTACNVGGGTATCTAATCC
	785R.8	R8	CAAGCAGAAGACGGCATACGAGAT	TTAACTGGAAGC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	CACTTCT	GACTACNVGGGTATCTAATCC
	785R.9	R9	CAAGCAGAAGACGGCATACGAGAT	TACCGCCTCGGA	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT		GACTACNVGGGTATCTAATCC
	785R.10	R10	CAAGCAGAAGACGGCATACGAGAT	ACTTTAAGGGTG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	A	GACTACNVGGGTATCTAATCC
	785R.11	R11	CAAGCAGAAGACGGCATACGAGAT	CCATCACATAGG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TC	GACTACNVGGGTATCTAATCC
	785R.12	R12	CAAGCAGAAGACGGCATACGAGAT	GAGCAACATCCT	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	CTA	GACTACNVGGGTATCTAATCC
	785R.13	R13	CAAGCAGAAGACGGCATACGAGAT	ATGTCCGACCAA	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GATA	GACTACNVGGGTATCTAATCC
	785R.14	R14	CAAGCAGAAGACGGCATACGAGAT	TGTCTCGCAAGC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ACTCA	GACTACNVGGGTATCTAATCC
	785R.15	R15	CAAGCAGAAGACGGCATACGAGAT	CGCGGTTACTAA	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TTCTCT	GACTACNVGGGTATCTAATCC
	785R.16	R16	CAAGCAGAAGACGGCATACGAGAT	GAGACTATATGC	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	CACTTCT	GACTACNVGGGTATCTAATCC

Table 2. Normalization of randomized DNA samples prior to and following amplification. Example worksheet listing samples in a randomized order and indicating their location on a single 96-well plate, which also determines the primer combination assigned to it. Formulas in the bottom row describe calculations for adding 100 ng of each sample to the normalized plate, plus the volume of water to reach 20 µL. Following amplification, the volume of 100 ng of each successful product is calculated and added to a final pool. The volume of "blank" PCR product to add to the final pool is the average of the other samples.

Sample	Sample name	Well	Forward Primer	Reverse Primer	DNA extract conc. (ng/µL)	Volume DNA for normalized plate (µL)	Volume H2O for normalized plate (µL)	PCR product conc. (ng/µL)	Volume for final pool (µL)
	BLANK	A1	341F_1	785R_1			20	6.76	4.00
1	RHIZO_1	A2	341F_1	785R_2	13	7.69	12.31	23.02	4.34
2	RHIZO_2	A3	341F_1	785R_3	18.6	5.38	14.62	21.84	4.58
3	SOIL_8	A4	341F_1	785R_4	4.2	23.81	-3.81	23.01	4.35
4	ROOT_22	A5	341F_1	785R_5	5.82	17.18	2.82	23.61	4.23
5	RHIZO_12	A6	341F_1	785R_6	7.72	12.95	7.05	26.87	3.72
6	SOIL_20	A7	341F_1	785R_7	30.2	3.31	16.69	18.76	5.33
7	ROOT_28	A8	341F_1	785R_8	5.39	18.55	1.45	28.40	3.52
8	SOIL_24	A9	341F_1	785R_9	11.1	9.01	10.99	25.02	4.00
9	ROOT_24	A10	341F_1	785R_10	7.79	12.84	7.16	23.48	4.26
10	SOIL_29	A11	341F_1	785R_11	6.98	14.33	5.67	26.36	3.79
	BLANK	A12	341F_1	785R_12			20	3.46	4.00
11	ROOT_1	B1	341F_2	785R_1	32.9	3.04	16.96	24.33	4.11
12	RHIZO_16	B2	341F_2	785R_2	14.6	6.85	13.15	25.03	4.00
.									
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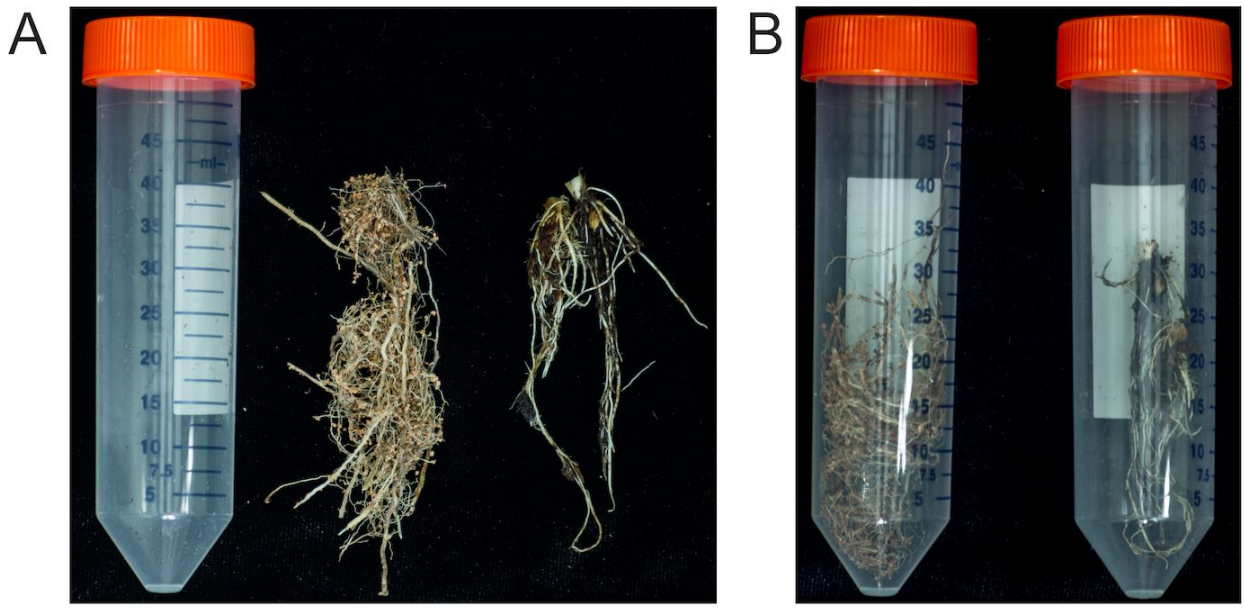
86	SOIL_2	H5	341F_8	785R_5	35.8	2.79	17.21	23.33	4.29
87	SOIL_12	H6	341F_8	785R_6	3.17	31.55	-11.55	32.52	3.08
88	SOIL_15	H7	341F_8	785R_7	7.85	12.74	7.26	25.27	3.96
89	ROOT_6	H8	341F_8	785R_8	10.1	9.90	10.10	28.18	3.55
90	RHIZO_11	H9	341F_8	785R_9	37.2	2.69	17.31	28.69	3.49
91	ROOT_31	H10	341F_8	785R_10	13.8	7.25	12.75	29.45	3.40
92	ROOT_32	H11	341F_8	785R_11	27.3	3.66	16.34	8.66	4.00
	BLANK	H12	341F_8	785R_12			20	10.76	4.00
Source of number or formula for calculations:					*Fluorometer*	*=100/(eDNA concentration)*	*=20-(volume DNA)*	*Plate reader or fluorometer*	*=100/(PCR concentration)*



Supplemental Figure 1.
Approximation of minimum root biomass during sample collection. When collecting roots, try to collect at least 500 mg of tissue. Here, roots collected from a young sorghum plant (left, in both A and B) and a young rice plant (right) are shown next to (A) and inside (B) 50 mL conical tubes. Both samples weigh approximately 1 g, however, it is important to note that this weight includes rhizosphere and root, and the rhizosphere weight is, in this case, approximately half the total weight.

B. Media recipes

ISP2

10g malt extract
4g glucose
4g yeast extract
15g agar
Add to 1L H2O. Autoclave. After autoclaving, add:
1mL vitamin B solution
10mL nystatin

Tap water-yeast extract (TWYE)

0.25g yeast extract
18g agar
0.5g K2HPO4
Dissolve in 1L tap water. Autoclave
After autoclaving, add:
5mL nystatin
1mL vitamin B solution

Skim milk (SM)

10g powdered skim milk
0.5g MgSO4
8g gellan gum (gelzan)
Dissolve in 1L H2O. Autoclave. After autoclaving, add:
1mL cycloheximide antifungal
1mL vitamin B solution
5mL nystatin

Humic acid (HA)

1g humic acid
0.5g Na2HPO4
1.71g KCl
0.5g MgSO4 * 7H2O
0.01g FeSO4 * 7H2O
0.02g CaCO3
8g gellan gum (gelzan)
Dissolve in 1L H2O
pH to 8.0 and autoclave. Then add
1mL cycloheximide antifungal
1mL vitamin B solution

Starch Casein Agar (SCA)

10g soluble starch
0.3g vitamin-free casein

2g KNO3
0.05g MgSO4 * 7H2O
2g NaCl
2g K2HPO4
0.02g CaCO3
0.01g FeSO4 * 7H2O
18g agar
Dissolve in 1L H2O. pH to 7
Autoclave
Add 10mL nystatin

M9 Media
Salt mixture
60g Na2HPO4
30g KH2PO4
5g NaCl
10g NH4Cl
Dissolve in 1L H2O and autoclave
Add 50mL salt mixture to 450mL H2O and 15g agar. Autoclave then add:
1mL MgSO4 (1M)
0.1mL CaCl2 (1M)
4mL glucose (50%)
0.25mL thiamine (1%)
0.5mL ampicillin (50mg/mL)

Vitamin B solution (1x)
0.5mg thiamine-HCl
0.5mg riboflavin
0.5mg niacin
0.5mg inositol
0.5mg Ca-pentathenate
0.5mg P-aminobenzoic acid
0.25mg biotin
Add to 10mL sterile H2O
Filter sterile