UNDERSTANDING THE MICROBIOME OF COMMON BEAN PLANTS (*PHASEOLUS VULGARIS* L.): DROUGHT STRESS, MICROBIOME TRANSMISSION AND ASSEMBLY, AND OUTLOOKS FOR AGRICULTURAL APPLICATIONS

By

Abby Grieb

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Microbiology and Molecular Genetics – Doctor of Philosophy
Ecology, Evolutionary Biology, and Behavior – Dual Major

2024

ABSTRACT

The plant microbiome is composed of diverse bacterial, archaeal, and fungal species that support the health of the plant in various plant compartments, including the root and surrounding soil, leaf surfaces, and internal tissues. These microbial species play an important role in plant health such as protecting the plant from pathogenic species and assisting with water and nutrient assimilation. With the ongoing climate crisis, it is important to understand how repeated seasons of stress, such as drought, are impacting the plant microbiome of agricultural crops, particularly staple food crops like common bean (dry beans). Work is being done to improve the resilience of common bean to environmental stress, including utilizing beneficial microbiome members to support the plant. However, the effect of drought on the common bean plant microbiome is not well understood, and it is important to select microbial inoculants that are also resistant to abiotic stress in the environment.

Chapter 2 describes a multigenerational experiment conducted to study the impact of repeated drought exposure to common bean over two plant generations and in two common bean genotypes, Red Hawk and Flavert. We identified more significant effects of the drought treatments and legacy effects in the microbiome of the Flavert plants, while the microbiome of Red Hawk was more stable. Additionally, we identified bacterial orders that are consistently associated with the drought treatment across generations and genotypes, particularly Xanthomonadales and Rhizobiales, which may contain target bacterial inocula for microbiome modification under drought stress. In Chapter 3 the seeds of the Red Hawk plants in the multigenerational experiment were investigated, with an additional treatment condition of increased fertilizer concentration. The aim of this study was to identify vertically transmitted

taxa in common bean seeds under abiotic treatment conditions. The stress treatments had a negligible effect on the resulting seed microbiomes, but we identified a significant impact of parental plant line and a signature of stable transmission of 22 prevalent seed microbiome members. These prevalent taxa included previously identified core taxa for common bean, and could be a valuable point of interest for the development of beneficial bacteria applications in agriculture.

Chapter 4 investigates the development of the rhizoplane and rhizosphere microbiome in common bean roots across plant growth stages. An innovative experiment utilizing a growth delay in common bean plants allowed the influence of plant growth stage and time across the common bean lifecycle to be investigated separately. We identified closely aligned bacterial communities in the rhizoplane of common bean based on growth stage in plants, despite differing growth rates. Indicator taxa associated with plant growth stages were identified and found to be under a selective pressure by the plant, and included known beneficial plant microbiome taxa.

This work provides important knowledge in understanding the impacts of repeated seasons of drought on plant microbiomes, exposes the importance of parental line in seed microbiome studies and the maintenance of the plant microbiome across generations, and provides insight into beneficial bacteria and the assembly processes of the plant microbiome. The need for sustainable solutions to support agricultural crops continues to rise, and this thesis contributes to our understanding of the processes shaping the beneficial plant microbiome that could be utilized in agricultural applications.

This thesis is dedicated to my grandmother, Mary Thomas.
My first teacher.

ACKNOWLEDGEMENTS

I am deeply grateful for the support and encouragement I received throughout my graduate school career that made this work possible. First, I would like to thank my PhD advisor, Dr. Ashley Shade. Ashley accepted me directly into her lab at the start of my PhD, and trusted me with a large and complex research project in my very first year as a PhD student. She has always been so supportive and helpful, even when conducting research was difficult during the COVID-19 pandemic. She has always supported all the extracurricular and career-development activities I have pursued and taught me how to be a great researcher and scientist. Ashley has been an incredible mentor over the past five years, and I am so grateful to have been able to work with her.

Additionally, I would like to thank my guidance committee, Dr. Matthew Schrenk, Dr. Greg Bonito, and Dr. Marjorie Weber, for their support, insight, and feedback as I completed my dissertation research. Marjorie also mentored me as an undergraduate researcher at Michigan State, and my experience doing research in her lab inspired me to pursue a PhD after completing my bachelor's degree.

I would also like to thank all the current and former members of the Shade lab who encouraged me and helped me complete my research projects. In particular, I would like to thank Dr. Fina Bintarti for teaching me how to grow beans in the growth chamber facilities and assisting with designing our multigenerational experiments, Keara Grady for her amazing help in showing me the ropes in the Shade lab and making sure everyone's science ran smoothly, and my undergraduate assistants, Bryce Davis and Joanna Colovas, who helped me keep my plants alive and complete the large growth chamber experiments. I could not have harvested all

those plants and done all the DNA extractions without their help. Additionally, I would like to thank Dr. Samuel Barnett for his help in completing my data analysis.

I was also fortunate in my graduate research to work with many amazing collaborators who assisted with experimental design and guidance on protocols for my work. I would like to thank Dr. Matthieu Barret, Dr. Marie Simonin, and Brice Marolleau from the INRAE in Beaucouzé, France. I had the wonderful opportunity to visit them at the INRAE in 2023 and learned so much from everyone there, especially Marie, who helped me with my data analysis skills. I have greatly enjoyed working with them on our collaborative project. Additionally, I would like to thank Dr. Chad Niederhuth and Hannah Cushman in the MSU Plant Biology department, who assisted with advice and materials for the zebularine experiment.

Outside of my research activities, I also had the opportunity to participate in many different organizations and career development activities. I would like to thank the MSU Plant Resilience Institute, which has been a wonderful group to be a part of and I have learned so much from the people I've been able to interact with through the institute. Also, I'd like to thank Julie Rojewski and the MSU BEST program for their helpful programming on non-academic career paths. I would also like to thank Dr. Robert Last and the Plant Biotechnology for Health and Sustainability Program for allowing me to join the program later in my PhD and giving me the opportunity to explore plant biotechnology and develop skills and interests for my future career. Additionally, I'd like to thank the Graduate Women in Science organization for all of the wonderful opportunities, and the Mid-Michigan chapter for their friendship and support throughout my time in graduate school.

Lastly, I would not have been able to complete my PhD without the love and support of my family and friends. Thank you to my wonderful parents, Bill and Patty Sulesky, for always supporting my dreams and encouraging me to be the best in all that I do. I will never forget my dad bringing me to visit Michigan State for the first time in 2013. I fell in love with MSU and that visit changed my life forever. Also, thank you to my in-laws, Chuck and Lynn Grieb, who have always been there for me and given me a home away from home for the past 10 years. Lastly, thank you to my husband, Thomas, for his endless support and encouragement. I could not have completed graduate school without him.

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CHAPTER 1:

Literature Review – Plant Microbiomes and their Role in Sustainable Agriculture

EXPLORING THE PLANT MICROBIOME

Microorganisms exist in every environment on earth, from the guts of animals and insects, to freshwater environments, to soils and subsurface sediments[1–5]. Microorganisms are ubiquitous in the environment and span a vast range of biodiversity[6,7]. It is likely that the known species of microorganisms currently documented account for only a small portion of the true biodiversity of bacteria, archaea, and fungi[8]. One area of particular interest in microbial ecology research is the plant microbiome. Plant microbiomes play an important role in plant health and provide many ecosystem services to their plant hosts and the environment at large[9]. Many bacterial, archaeal, and fungal species can inhabit the plant in various plant compartments and there is considerable work being done to investigate the membership of plant microbiomes in a wide range of plant species and environments[10]. Understanding key members of the microbiome is not only useful ecologically, but also allows researchers to harness the beneficial properties of these microbes for use in agricultural and environmental applications.

Plant microbiome diversity and characteristics

Microorganisms are found in all compartments of the plant (the various tissues and structures that make up the plant) and require unique traits and life strategies to survive in these environments. The most diverse community associated with the plant is the rhizosphere. The rhizosphere is defined as the soil directly surrounding the roots that is impacted by the plant, as compared to bulk soil communities that are not directly affected by the plant[11]. The rhizosphere environment can contain a wide range of microorganisms, including bacteria, archaea, and fungi, and larger organisms like nematodes and small insects[12,13]. The rhizosphere can contain tens of thousands of microbial species, with some estimates stating that there are

over 50,000 species of microbes in one gram of soil[14]. There are often tradeoffs between microorganisms in different niches in these communities, where one species may produce a byproduct that is consumed by another, and species utilize a wide range of energy sources[15–17]. The rhizosphere is also susceptible to dynamic changes in resources and water availability, and microbial species must be able to adjust to their ever-changing environment[18]. Many factors can influence the microbial community in the rhizosphere, including soil moisture and abiotic properties, the presence of other organisms like protists, and the progression of the plant over time[19–23]. Rhizosphere communities include many taxa that are categorized as beneficial to the plant, including plant growth promoting bacteria, nitrogen fixing rhizobia, mycorrhizal fungi, and bacteria that provide biocontrol of pests[24–27].

Another compartment of the plant microbiome associated with roots is the rhizoplane. The rhizoplane community is defined as the microbial species that are closely associated with the surface of plant roots, and can be more or less diverse than the rhizosphere depending on the plant and environment[28–31]. These microbial species are under greater influence from the plant roots at small scales, where species are recruited to the rhizoplane via plant-microbe feedbacks and further symbioses can develop[32,33]. The composition of the rhizoplane is similar to the rhizosphere, but often contains more beneficial microbes over commensal organisms and increased functional diversity[30]. Microbial species in the rhizoplane often include bacteria from the phyla Firmicutes, Proteobacteria, Actinobacteria, and Bacillota, among others, as well as fungal taxa[34–38].

The plant microbiome also contains many endophytic taxa, which are microbial cells living inside plant tissues both intercellularly and intracellularly[39,40]. Endophytic microbes can be

found throughout the plant in all plant structures, such as stems, leaves, vascular tissues, and seeds, and are typically less diverse than those living in the soil[39–41]. Plant tissues and intercellular spaces can have highly variable nutrient availability and present difficult living conditions for bacteria[42]. Many endophytic species typically have unique traits that allow them to colonize the plant more easily, such as lipopolysaccharides and motility which assist with active colonization, and plant cell-wall degrading enzymes which can enable passive colonization of plant tissues[39].

Many rhizosphere and rhizoplane taxa are commonly found inside the plant root as endophytes, and are typically more specialized and provide benefits to the plant[28,42–44]. Root endophytes can colonize the root tissues through the root tips, hairs or cracks at secondary root emergence sites[39]. Stem and vascular tissue endophytes are often closely linked to root communities, and these compartments can serve as a colonization route for bacteria as water moves throughout the plant[39]. Endophytes are also found within the leaf and seed tissues of the plant. Seed endophytes can be linked to the microbial community in the vascular system and can also be deposited by pollinators through floral compartments[45,46]. Seeds are a particularly challenging environment for microbial species to inhabit because they become dormant at maturity[47–49]. Seed endophytes need strategies to survive in dormant seeds, such as sporulation, desiccation stress tolerance and dormancy of their metabolic activities while nutrients are sparse[50]. Seeds typically harbor the lowest microbial diversity within the plant microbiome, containing tens to hundreds of taxa[51].

Finally, the phyllosphere microbiome is comprised of microbial species that colonize the surfaces of the plant that are impacted by environmental factors[10]. Bacteria living in the

phyllosphere need additional strategies that allow them to survive there, including desiccation, heat and UV stress tolerance, and specialized metabolisms for low-nutrient environments[52]. The phyllosphere is the harshest of the plant microbiome environments, but contains a wide diversity of species that may be selected for by the plant or deposited by stochastic processes such as irrigation and wind[53–56]. Plants may also contain specialized structures on their surfaces that harbor unique microbial communities, such as aerial roots or epicuticular wax[57]. Overall, the plant microbiome is a highly diverse environment with many specialized species that have adapted to thrive both on and inside of plant compartments.

Beneficial properties of the plant microbiome

The bacterial and fungal species living in and on plants can include commensal organisms that utilize resources provided by the plant without affecting the plant in any way, pathogenic species that can cause harm to the plant, or beneficial species that provide a wide range of valuable properties that support plant growth[58,59]. There are many ways that beneficial species can support the plant, including providing protection from pathogenic species, providing valuable nutrients to the plant, releasing plant growth-promoting factors, or assisting with water uptake and drought resistance. For example, *Pseudomonas* strains have been shown to be effective in controlling blight in potato, and the co-inoculation of common bean with mycorrhiza and *Rhizobium* symbionts increased plant tolerance to Fusarium root rot[60,61]. Many studies have also found that the inoculation of beneficial bacteria in root systems increases nutrient uptake from the soil in plants such as tomato, soybean and common bean[62–64]. The hyphal structures of mycorrhizal fungi are also known to play a role in water uptake by roots, and inoculants of beneficial bacteria and fungi can improve plant growth under drought

stress[65,66]. As discussed, these beneficial microbes contribute to the health of plants in a wide range of environments. This area of research is particularly useful in agricultural applications, and there is a growing literature investigating the use of beneficial microbes for inoculations in the field to replace exogenous irrigation, fertilizer, and pesticide use.

PLANT MICROBIOME ASSEMBLY AND INTERACTIONS

Horizontal assembly and plant-soil feedbacks

Plant microbiome members can be assembled in the microbiome through a variety of processes. Horizontal assembly involves microorganisms colonizing the plant from external sources such as soil, water, and air[55]. Soil microbes can disperse to above and below-ground plant compartments via stochastic processes and are also enriched and recruited by the plant in these environments[37,67–69]. As plants are growing, they release chemicals such as phytohormones and produce immune responses that can enrich or modulate species of bacteria and fungi[70–72]. These plant-microbe feedbacks play an important role in shaping the microbiome, particularly in root communities, and communities can shift dramatically over time[73–75]. It has been demonstrated that root exudates such as malic acid recruit beneficial rhizobacterium species in *Arabidopsis*, and other organic acid exudates are linked to bacterial responses over the wheat lifecycle[76,77]. Changes in root exudation have also been investigated for various plant species under altered environmental conditions, such as in maize and locust trees[20,78]. In addition to horizontal accumulation of taxa on plant surfaces and in roots, microbiome members can be deposited into floral compartments which can impact the establishing seed microbiome[46,79]. Microbiome members can also be stochastically deposited

on the surface of seeds as seeds are dispersing from the parent plant, which can become available in the next plant generation as seeds germinate[80].

Vertical transmission

In contrast to recruitment from the environment, there is also evidence of vertical transmission of microbiome members from parent plants to offspring through seeds and clonal plants[80–85]. As seeds are developing in the parental plant, endophytic microbiome members can assemble within the seed coat in the internal tissues of the seed embryo and endosperm[80]. This has been documented in many plant species and can happen through multiple pathways, such as through the vascular tissue of the parent plant in common bean and rice, or through floral compartments as has been demonstrated in other cultivated and wild species[45,46,86–88]. The microbiome members that are packaged within seed compartments become the starting point for the microbiome of germinating seedlings, and priority effects may allow them to establish in the plant microbiome before horizontally acquired species[80,89–92]. Microorganisms that are vertically transmitted through seeds have been shown to possess plant-beneficial properties and may be key taxa for the health of the next plant generation[93–97]. Additionally, plants that reproduce through clonal mechanisms have been documented as passing microbiome members from parent plant to offspring through vertical transmission[85,98]. Both the horizontal transmission and assembly from the environment through plant-soil feedbacks, and vertical transmission from parent plant to offspring, can play a major role in shaping the plant microbiome.

Symbiotic relationships within plant compartments

While assembly processes of the plant microbiome can accumulate various commensal or harmful microbial taxa to plant compartments, plants often recruit specific microbiome members that provide important beneficial functions in the microbiome ecosystem. These plant-microbe interactions are known to enhance the growth of the plant and perform important ecosystem functions.

One example of an important plant-microbe symbiosis is the formation of root nodules in leguminous plants. Specialized signals between the plant roots and diazotrophic bacteria in the family Rhizobiaceae regulate the process of root nodule formation, allowing bacteria to invade the root via root hairs[99,100]. Once inside the root, nodule structures form around the bacteria and the rhizobia species perform biological nitrogen fixation, which converts dinitrogen gas (N_2) from the air into ammonia (NH_3) which can be utilized by the plant[101]. Plant species often recruit specific rhizobia symbionts, but nodules can also contain diverse non-rhizobial endophytes[102–105]. The fixation of nitrogen by rhizobia in legume root nodules also provides biologically available nitrogen for other plant species, and rotational cropping with legumes is an important aspect of modern agriculture to maintain nitrogen fertility in agricultural soils[106]. Free living bacteria are also capable of performing biological nitrogen fixation, and diazotrophic rhizobia species are being investigated for use as inoculants to improve nitrogen availability not only in legumes, but other crops as well[107–111].

Further, symbioses with mycorrhizal fungi also play an important role in the plant microbiome[25,112]. The most prevalent mycorrhizal fungi in agricultural systems are arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF), and these fungal symbionts are

present in almost all major groups of plants[25]. Associations of mycorrhizal fungi with plants has evolved across multiple lineages of fungi, and fungal symbionts play an important role in the health and stress tolerance of plants[112–115]. Naturally occurring symbioses are highly important to plant growth and survival under stress conditions, and these relationships are being investigated for exploitation in agricultural applications.

Recruitment of core taxa

Many microbial species have been documented as "core" taxa for various plant species. Core taxa have had many different definitions in the literature, such as taxa that are highly abundant, present in all samples, or enriched in a condition of interest[116]. These definitions have been used to investigate core taxa in a wide range of plant microbiomes, including *Arabidopsis*, common bean, poplar, maize and sugarcane[36,117–120]. Core taxa in these studies are typically consistently identified in the plant microbiome based on abundance, occupancy, or a combination of methods, and in some studies are found associated with the same plant species despite being grown in different locations[117,121]. Since a hallmark of core identification is consistent detection in the plant microbiome, it is likely that these taxa provide a benefit to the plant. However, the mechanism of core taxa association is not fully understood, and horizontal or vertical assembly processes are both possible as discussed previously. Additionally, seed microbiomes have been identified as potential reservoirs of core taxa in a variety of plant species[122–125]. Regardless of recruitment method, it is likely that these microbial species play an important role in plant health, and may be under a selective pressure from the plant as root-associated or endophytic taxa. The core plant microbiome is of particular interest as a target for beneficial crop treatments with microbiome modification.

APPLIED RESEARCH IN PLANT MICROBIOMES FOR AGRICULTURAL APPLICATIONS

Current threats to global agriculture

The exponential growth of the human population combined with anthropogenic climate change has created a crisis for current agricultural systems. While the global food demand continues to grow, arable land area is decreasing, and climate change is significantly impacting agricultural production. Deforestation to convert landscapes to farmland is not a viable option, as it contributes to the climate crisis with the loss of forests as carbon sinks and increased land temperature[126]. Extreme weather events are also on the rise, particularly hydrological events such as flooding and drought[127]. It is estimated that dry growing seasons reduce the yield of major crops in Australia by 25-45%, and a meta-analysis of 30 plant species found significant negative impacts of drought and high temperatures on crop yield and seed quality[128,129]. Irrigation during drought and applications of artificial fertilizers also contribute to climate concerns, particularly with greenhouse gas emissions from fertilizer production[130]. Developing countries are at exceptionally high risk of negative climate change impacts[131,132]. Many people in rural areas are dependent on subsistence farming, which is being threatened by changing temperatures, extreme weather events, droughts, soil degradation and pests and disease[133–135]. As these challenges to agriculture persist, it is important to find sustainable solutions that increase crop yield without contributing further to the climate crisis.

Effects of abiotic stress on the microbiome

Climate change not only has a profound effect on agricultural systems, it also significantly impacts the microbial life on our planet. Soil microbiomes contribute to biogeochemical cycling, and changing environmental conditions will result in significant impacts

on these processes, particularly in ecosystems that are more sensitive to climate change[4]. It has been estimated that current climate projections could result in significant declines of beneficial plant microbiome members, particularly bacteria that assist with biocontrol of pests and stress resistance[136]. The plant microbiome is important for plant resilience against changing climate conditions, and it will be critical to understand how these microbial communities are impacted by environmental conditions in the coming decades[137]. While there are many knowledge gaps in this area, some work has been done to investigate the impacts of abiotic stress on plant microbiomes. Abiotic stress conditions such as drought can affect root exudation in plants, which in turn has been demonstrated to affect the development of root microbiomes in maize, sorghum and rice[138–141]. The phyllosphere and endosphere can also be altered by abiotic stress such as drought affecting the seed microbiome of common bean, or extreme heat altering floral communities in wildflowers[79,142,143]. This area of research continues to grow, and understanding the impacts of changing climate conditions on plant-microbe interactions will provide important knowledge to the field to improve plant resilience under stress[144].

Potential solutions harnessing the plant microbiome

While changing climate conditions is an area of significant concern for agriculture, there is also noteworthy research being done to find sustainable solutions that harness beneficial microbes to support agricultural crops[145,146]. Utilizing microbial inoculants to improve plant health and resistance to stress and disease has been investigated in a wide range of plant systems, including barley, soybean, grapevine and many others [62,63,66,147–149]. It has been demonstrated that applications of bacteria or introductions of native microbial consortia can assist plants in responding to altered environmental conditions[150,151]. These processes are being

widely investigated to exploit for the support of agricultural crops in commercial applications[152–155]. Further, as evidence for plants enriching beneficial microbiomes increases, researchers are now considering the plant microbiome when breeding new varieties of crops[156,157]. Application methods of beneficial microbes, such as seed treatments and foliar sprays, are also a burgeoning area of research, as timing and persistence of inoculants in the plant microbiome are also important considerations when determining their effectiveness[158–160]. Finding solutions to the challenges being faced by agricultural systems is of utmost importance for the future of food security. Although much work is being done in this area, there are also significant knowledge gaps around which microbial species are the best to use for inoculations, and how these species should be applied in the field[161].

STUDY SYSTEM - COMMON BEAN

This work focuses on the microbiome of the plant species *Phaseolus vulgaris* L., also known as common bean or dry beans in agricultural contexts[162]. Beans were domesticated around 8,000 years ago in two different regions in central and South America, and have been an important food crop for centuries[163,164]. In 2020, there were 34.8 million hectares of beans harvested around the world, and production continues to increase annually[165]. Developing regions rely on dry beans as a major food crop, as they are nutrient dense and dormant beans can be stored for long periods of time[47,166].

Climate impacts on dry bean production may be severe in coming years, and research is being done in plant breeding, genetics, and microbiome amendment to improve the growth of common bean under stress conditions[126,167,168]. Investigating the specific rhizobia species that nodulate dry bean has been a growing area of research, with studies looking to utilize rhizobia

from primitive common bean landraces as a source for potential inoculants of agricultural varieties[169,170]. Other studies have tested various inoculants to improve the growth of beans under abiotic stress [110,171–173]. The work presented here investigates the impact of environmental stress on the root and seed microbiome, and the root microbiome development of the kidney bean variety "Red Hawk", in order to contribute to the growing literature of potential uses for the plant microbiome to support important agricultural systems[174].

SUMMARY AND RESEARCH AIMS

Research on the beneficial properties of plant microbiomes is a growing field, and many studies have investigated the identity and potential functions of beneficial bacteria and fungi in plant microbiome compartments. Microorganisms play an important role in plant growth promotion, protecting plants against pathogens, and improving water and nutrient assimilation in plant roots. The microbiome can be assembled both horizontally from the environment and through vertical transmission from parent plants, and plants are able to recruit specific microbial taxa to their microbiome that likely benefit the health of the plant. While this has been an exciting area of research over the past few decades, many questions remain about the impacts of changing environmental conditions on the plant microbiome and how the microbiome can be utilized in sustainable agriculture systems.

This thesis will investigate the microbiome of common bean plants, *Phaseolus vulgaris* L., in the context of drought stress, and the development of the root microbiome over the lifecycle of the plant. Although both bacterial and fungal species are important in plant microbiomes, this work focuses on the bacterial portion of these communities. In chapter 2, a multigenerational experiment will be described where two common bean varieties were

exposed to drought stress over two plant generations, and the root and rhizosphere microbiomes were assessed to address the impacts of repeated stress exposure on the microbiome. Chapter 3 investigates the seeds of the plants in the multigenerational experiment, with an additional treatment condition of increased fertilizer concentration. The aim of this study was to identify vertically transmitted taxa in common bean seeds under abiotic treatment conditions. Lastly, chapter 4 investigates the development of the rhizoplane and rhizosphere microbiome in common bean roots across plant growth stages. The overarching goal of this work is to identify bacterial taxa that are responsive to plant stress or are consistently found in common bean, and propose some target species for use in agricultural applications. This thesis will contribute to the growing area of research in beneficial plant microbiomes and will provide important knowledge for the development and application of beneficial microbial treatments in the field.

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CHAPTER 2:

Belowground plant microbiome resistance and response after two generations of drought exposure in common bean

ABSTRACT

Climate change is a topic of significant concern for the global food supply, and agricultural crops are experiencing unprecedented environmental conditions such as flooding, heat stress and drought. Drought stress can have severe impacts on crops, particularly in developing countries where people rely on staple food crops like common bean (*Phaseolus vulgaris* L.). Work is being done to improve the resilience of common bean to environmental stress, including utilizing beneficial microbiome members to support the plant. However, the effect of drought on the common bean plant microbiome is not well understood, and it is important to select microbial inoculants that are also resistant to abiotic stress in the environment. In this study, we conducted a multigenerational experiment to study the impact of repeated drought exposure to common bean over two plant generations and in two common bean genotypes, Red Hawk and Flavert. We identified more significant effects of the drought treatments and legacy effects in the microbiome of the Flavert plants, while the microbiome of Red Hawk was more stable. Additionally, we identified bacterial orders that are consistently associated with the drought treatment across generations and genotypes, particularly Xanthomonadales and Rhizobiales, which may contain target bacterial inocula for microbiome modification under drought stress. This work provides important knowledge in understanding the impacts of repeated seasons of drought on plant microbiomes and potential taxa to investigate for plant microbiome engineering.

INTRODUCTION

Environmental changes are becoming an increasingly important factor in today's agricultural systems[1–3]. Changing weather patterns have resulted in severe drought in many regions of the world, which are particularly devastating in developing countries facing severe food insecurity[4–8]. An important and growing area of research is focused on the impact of these environmental changes on agricultural crops, with particular interest in the plant microbiome[9–12]. The plant microbiome comprises millions of bacterial, archaeal, and fungal cells that inhabit the plant's surfaces, tissues and root structures[13,14]. The beneficial microbiome plays an important role in plant health and provides many important services in agricultural systems, including water and nutrient assimilation to the plant, nutrient cycling in the soil and root system, and pathogen resistance[13–16].

Many studies have been conducted to improve our knowledge of the plant microbiome and its role in agriculture for various crops[17–19]. While some studies in recent years have addressed the impacts of environmental stress on the microbiome of plants, few have studied this over multiple generations of repeated stress exposure[20–26]. With the continuing trends of anthropomorphic climate change, repeated exposure to drought conditions each growing season will become common for many agricultural areas. These repeated stress conditions over multiple growing seasons may create unique challenges for crop plants, such as negative impacts on seed quality and long-term alterations of soil conditions[1,2]. Our study aimed to understand the impacts of repeated drought exposure on the microbiome of common bean (*Phaseolus vulgaris* L.).

Common beans are a vital food source around the world, with 27.5 million metric tons of food produced annually and cultivated across 34.8 million hectares of land as of 2020[27,28]. The common bean species, *Phaseolus vulgaris* L., includes a wide range of bean varieties that are considered "dry edible bean" and fresh "garden bean" crops, including kidney beans, black beans, navy beans, green beans, and others[29]. These varieties have been bred over 8,000 years of domestication from two ancestral lines originating in Mesoamerica and the Andes Mountains[27,30]. Beans not only provide valuable nutrition, but also perform important symbiotic nitrogen fixation that provides usable nitrogen to other crops[31]. These services are particularly valuable in developing countries that are most greatly impacted by environmental stress, due to a lack of infrastructure to manage poor growing conditions and reliance on subsistence farming, and the ability to continue to produce dry beans in these areas is of vital importance[32].

Work has been done to improve the drought tolerance of common bean through breeding and plant genetics, but utilizing the plant microbiome to confer stress tolerance is a growing area of research[33,34]. Additionally, various studies have been conducted to understand the bean microbiome, bean plants' association with nitrogen-fixing rhizobia, and the impact of abiotic stress, such as drought, on the bean microbiome[35–42]. However, few studies have investigated the impact of repeated stress exposure on the microbiome of common bean or other crops[43]. The study presented here will contribute to our understanding of how plant microbiomes can be affected by stress over generations, and may provide insights into potential management strategies that harness the benefits of the plant microbiome.

We conducted a multigenerational experiment exposing common bean plants to drought stress in agricultural soil under controlled growth chamber conditions. We

hypothesized that (1) the bean plant health would be impacted by the drought treatment, (2) the microbiome of the plant roots and rhizosphere would be altered by the drought treatment in one or both generations in either alpha or beta diversity measures, and (3) that repeated exposure to drought over two generations would have consequences, either positive or negative, for plant health in the second generation. Specifically, we predicted that exposure to drought in the first generation may enrich beneficial microbiome members that could improve plant outcomes under drought when passed to the second generation. Alternatively, plant outcomes could be negatively impacted by repeated drought exposure. We tested our hypotheses in two different genotypes of *Phaseolus vulgaris* L., the Red Hawk variety, a representative kidney bean cultivar bred in North America, and Flavert, a representative European bean cultivar, in order to assess the impact of drought on the species broadly[44–46]. We also conducted the experiment in two geographic locations, Pays de la Loire, France, and Michigan, USA, with agricultural soil from these regions, in order to understand if the region and soil play a role in how the microbiome responds to stress. Common beans are regularly produced in both of these regions. Since the experiments and DNA sequencing were performed separately for the two locations with some differing parameters (see methods), the datasets were analyzed independently and potential differences between the datasets were expected.

The multigenerational experiment consisted of seeds provided from a "Generation 0 (zero)" seed pool planted in a controlled growth chamber and exposed to either a control condition with ample water and nutrients, or a drought condition with 60% decreased water but equivalent nutrients provided (Figure S1.1). Seeds from these "Generation 1" (G1) plants were harvested and used for growth in "Generation 2" (G2), where plants were exposed to

stress in a factorial design, either receiving the same or opposite treatment in generation 2. The plant growth, yield, and 16S V4 rRNA gene amplicon sequencing of the root and rhizosphere microbiome compartments were assessed in order to understand the impact of the first- and second-generation drought treatments on the plants and their root microbiome community.

METHODS

Bean cultivars

Two cultivars of common bean were used to complete this experiment. *Phaseolus vulgaris* L. var. Red Hawk, developed by the Michigan State University Bean Breeding program, was selected as a representative dry bean crop[46]. *Phaseolus vulgaris* L. var. Flavert was selected as a representative flageolet bean variety common in French agricultural production[45]. Red Hawk seeds in the Michigan location were obtained from the Michigan State University Bean Breeding Program from their 2019 harvest and stored at 4°C until ready for use in experiments. Flavert and Red Hawk seeds in the France location were purchased from Vilmorin-Mikado (Limagrain group, France). These seeds were obtained as “Generation 0 (zero)” and used to plant the first generation of the experiment.

Soil preparation

For growth experiments performed in Michigan, agricultural field soil was collected from the Michigan State University Agronomy Farm from a field that had previously grown common bean in 2019 (42°42'57.4"N, 84°27'58.9"W, East Lansing, MI, USA). The soil was a sandy loam with an average pH of 7.2 and organic matter content of 1.9%. Soil was collected before each planting group from the same field location. Five-gallon buckets were used to collect enough soil needed for the planting experiment, avoiding the dry top layer of soil and plant debris.

Buckets of soil were covered with air-tight lids and stored at 4˚C until ready for use in the growth experiment. Immediately before use in planting, soil was passed through a 4mm sieve to remove rocks and plant debris, and the soil was mixed with autoclaved coarse vermiculite at a 50% v/v ratio for use in growth chamber pots.

For Generation 1 experiments performed in Pays de la Loire, soil was collected from the experimental station of the National Federation of Seed Multipliers (FNAMS, 47°28'012.42"N – 0°23'44.30"W, Brain-sur-l'Authion, France) where common bean had been cultivated in 2016. This soil was a clayey sand limestone with pH 7.1 and 1.9% organic matter content. Soil was sieved and mixed with coarse vermiculite in the same method as above. Soil materials for Generation 2 were collected from the experimental fields belonging to the Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE) in Angers, France (47°28'50.7"N, 0°36'31.4"W). A different soil was used in G2 to simulate a realistic scenario during which seed multiplication occurs at a different site (seed producer) than the bean production (farm). This soil had a sandy-loam texture with a pH of 6.5 and 1.9% organic matter content. Soil was sieved and mixed with vermiculite in the same fashion as G1.

Surface sterilization and seed germination

For each planting group in Michigan, Red Hawk seeds were surface sterilized prior to planting with a solution of 10% bleach and 0.1% Tween20. Seeds were randomly selected from the bulk Gen0 seed supply, or the harvested Gen1 seed supply, avoiding seeds that were visibly cracked or moldy, and 20 seeds were placed in a sterile 50 mL conical tube. 20-30 mL of bleach solution was added to the tube and the seeds were left to soak for 10 minutes, swirling halfway through. After soaking, the seeds were rinsed 5 times with sterile DI H_2O. On the final rinse,

approximately 100 μL of rinse water was spread onto TSA (Tryptic Soy Agar) and PDA (Potato Dextrose Agar) plates for sterility assessment. TSA plates were incubated overnight at 28°C, and PDA plates were incubated at room temperature for 48 hours. Seeds corresponding to plates with microbial growth were discarded. After the final rinse water was plated, seeds were placed in a Petri dish lined with sterile filter paper. 1-2 mL sterile DI H_2O was applied to the filter paper and Petri dishes were stored in the dark at room temperature for 3-4 days for seeds to germinate, with an additional 2 mL sterile DI H_2O added halfway through the germination period. Once seeds had sprouted radicle roots they were transferred to soil for growth.

Seeds planted for growth in Pays de la Loire were not surface sterilized prior to planting, and were germinated directly in the prepared pots of soil. The experiment in Pays de la Loire included both Red Hawk and Flavert cultivars under the same soil and treatment conditions for each generation.

Growth conditions

For Generation 1 in Michigan, 1-gallon pots were filled with the soil-vermiculite mixture and three germinated seeds were planted per pot in a high-light BioChambers FLEX™ LED growth chamber with a 16-hour day/8-hour night cycle at 26°C and 22°C, respectively, and 50% relative humidity. Once seedlings emerged and reached the VC growth stage (two cotyledons and primary leaves expanded), they were culled to one seedling per pot. Plants were watered every other day with 300 mL 0.05% 15-10-30 water-soluble fertilizer solution (control condition) (Masterblend International, Morris, IL, USA). At the V3 stage (third trifoliate leaves expanded), stress treatments began for the drought treated plants. Drought plants received 100 mL of 0.15% 15-10-30 fertilizer solution every other day (66% less water than control with

the same concentration of nutrients). After approximately 14 days of treatment period, when plants reached the R1 stage (first open flowers), they were returned to the control watering every other day until senescence. As plants began to dry, they were watered less frequently as needed. There were 20 plants grown per treatment in G1. Five plants per treatment were destructively harvested for plant phenotypic measurements at the R5 stage (most pods at the seed filling stage), 5 plants per treatment were used for root and rhizosphere microbiome analysis at senescence, and mature seeds were collected from 12 plants per treatment for planting in G2. Seeds from the additional plants were harvested and stored as reserve.

For Generation 2, seeds from the 24 G1 parental lines that received either control or drought conditions were planted in a full factorial design and grown under both treatment conditions in G2. There were four treatment combinations total, Control_Control, Control_Drought, Drought_Control, and Drought_Drought (G1_G2). Eight seeds from each parental line were surface sterilized and germinated as above, then planted in the field soil-vermiculite mixture in seedling trays in the growth chamber under the conditions stated above. Once plants reached the VC stage, four viable seedlings per parent line were transferred to 1-gallon pots for the remainder of the experiment. Each parental line had two offspring treated with each of the treatments. One of those two offspring was used for plant phenotypic measurements while the other was harvested for microbiome analysis. Plants were watered according to the conditions and treatment timeline in G1, above.

Plants grown in Pays de la Loire were grown under the same growth chamber, control and drought conditions above. The experiment in Pays de la Loire was replicated with both Red Hawk and Flavert seeds. After three weeks of growth, day 18 after sowing (V3 stage), replicate

plants (n= 5) were exposed to control treatment (300mL of 0.05% nutritive solution) or drought stress (66% water-withholding, 100mL of 0.15% nutritive solution) for a period of four weeks until Day 56, the R5 growth stage (pods at 8-10 cm long with discernible seeds), a slightly longer stress period than applied to the plants in Michigan. Five plant parental lines from each G1 condition (10 lines total) from both genotypes were planted in Generation 2 in Pays de la Loire, and were also planted in a full factorial design with four treatment conditions in G2. See figure S1.1 for experimental design schematic.

Plant phenotypic data collection

Plants dedicated for phenotypic trait measurements at MSU were analyzed with a LI-COR LI-6800 instrument to measure photosynthetic rate and stomatal conductance the day before the stress treatment period ended to assess the impact of the treatment condition (LI-COR Biosciences, Lincoln, NE, U.S.A). Plants were then grown until they reached approximately the R6 growth stage where pods were developed with discernable seeds, but pods were green and were not yet drying. Pods were removed and placed in a paper bag, plants were cut at the base of the stem and shoots were placed in a large envelope. Roots were removed from the pot, shaken to remove excess soil, collected, rinsed, and then placed in a separate paper bag. Pods and seeds per plant were counted, and then all three compartments were placed in a 50 °C drying oven for two days. After drying, the shoot, root and pod dry biomass was measured.

In Pays de la Loire, plants were harvested for trait measurements at approximately the R5 stage. The entire root system was gently separated from the soil in the pot and placed in a plastic bag. The total fresh weight of each plant (both above- and below-ground tissues) was measured and the number of pods and seeds per plant were counted.

Microbiome compartment harvest

Once the Michigan plants had senesced and pods were dried, plants were harvested for microbiome analysis. Seed pods were removed and stored in a sterile Whirl-pak® bag. Root systems were removed from the pot and shaken to remove loose soil. Roots were collected in a Whirl-pak® bag and associated rhizosphere soil was collected in a sterile 50 mL conical tube. Prior to analysis, pods and seeds per plant were counted, and then seeds were removed from the pods and pooled by plant in 50 mL conical tubes and were stored at 4°C for planting. Roots and rhizosphere soil were stored at -80°C until further analysis.

The microbiome analysis in Pays de la Loire was performed on fresh plants harvested at approximately the R5 stage. The entire root system was gently separated from the soil in the pot and placed in a plastic bag. The rhizosphere soil was collected by shaking the root system in a plastic bag and stored at -80°C. The root system was then washed in sterile distilled water and transferred to 50 mL tubes and stored at -80°C.

DNA extractions

The Michigan root samples were thawed at room temperature and a 1-2 inch section of the main root system was cut and used for root DNA extraction which combined the rhizoplane and endophytic bacteria inside the root tissues. The selected sections were briefly rinsed in sterile DI water then placed in a clean mortar. Liquid nitrogen was added to the mortar and the roots were finely ground with a pestle. The ground root material was transferred to a 1.5 mL microcentrifuge tube and used for DNA extraction with the DNeasy PowerSoil Pro DNA Kit (Qiagen, Germantown, MD, USA) following manufacturer's instruction with the following modifications. In step one, 750 µL solution CD1 was used with 50 µL ATL buffer (Qiagen,

Germantown, MD, USA). The bead beating step was performed for 15 minutes on a vortex genie 24-tube adapter at maximum speed. Lastly, 60 µL of the final elution buffer C6 was used, and tubes were incubated for 10 minutes before centrifugation.

Rhizosphere soil was thawed at room temperature and DNA extracted with the DNeasy PowerSoil Pro DNA Kit with the same ATL buffer variation as the roots, above. Negative controls were included with each batch of DNA extractions from the start of the extraction procedure, and one positive mock community control was included with each compartment sample set (roots or rhizosphere)[47]. Control extraction samples were sequenced with the experimental samples for use in data processing.

French samples were processed in the same fashion and extracted using the DNeasy PowerSoil Kit (Qiagen, Germantown, MD, USA, *discontinued*) following manufacturer's instructions. A blank extraction kit control, a PCR-negative control and PCR-positive control (*Lactococcus piscium*, a fish pathogen that is not plant-associated) were included in each PCR plate.

Sequencing

Sequencing of the 16S V4 rRNA gene for the Michigan root and rhizosphere samples was performed at the Argonne National Laboratory Environmental Sample Preparation and Sequencing Facility (Lemont, IL, USA). The DNA was PCR amplified with region-specific primers that include sequencer adapter sequences used in the Illumina MiSeq ; FWD:GTGYCAGCMGCCGCGGTAA; REV:GGACTACNVGGGTWTCTAAT [48–52]. Each 25 µL PCR reaction contained 9.5 µL of MO BIO PCR Water (Certified DNA-Free), 12.5 µL of QuantaBio's AccuStart II PCR ToughMix (2x concentration, 1x final), 1 µL Golay barcode tagged Forward

Primer (5 µM concentration, 200 pM final), 1 µL Reverse Primer (5 µM concentration, 200 pM final), and 1 µL of template DNA. The conditions for PCR were as follows: 94 °C for 3 minutes to denature the DNA, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; with a final extension of 10 min at 72 °C to ensure complete amplification. Amplicons were then quantified using PicoGreen (Invitrogen) and a plate reader (InfiniteÒ 200 PRO, Tecan). Once quantified, volumes of each of the products were pooled into a single tube, in equimolar amounts, this pool was then cleaned up using AMPure XP Beads (Beckman Coulter), and then quantified using a fluorometer (Qubit, Invitrogen). After quantification, the pool was diluted to 2 nM, denatured, and then diluted to a final concentration of 6.75 pM with a 10% PhiX spike for sequencing. Amplicons were sequenced on a 251bp x 12bp x 251bp MiSeq run using customized sequencing primers and procedures [50].

For the 16S V4 rRNA gene sequencing in Pays de la Loire, PCR reactions were performed with a high-fidelity Taq DNA polymerase (AccuPrimeTM Taq DNA Polymerase System, Invitrogen) using 5µL of 10X Buffer, 1µL of forward and reverse primers (10µM), 0,2µL of Taq and 10µL of DNA. A first PCR amplification was performed with the primer sets V4 515f/806r (5'-GTGCCAGCMGCCGCGGTAA-3'and 5'-GGACTACHVGGGTWTCTAAT-3'[49]. Cycling conditions were composed with an initial denaturation at 94°C during 3 minutes followed by 35 cycles of denaturation at 94°C (30 seconds), primer annealing at 55°C (45 seconds) and extension at 68°C (90 seconds), with a final elongation at 68°C for 10 minutes. Amplicon purification was performed with a ratio at 0.8 of magnetic beads (Sera-MagTM, Merck). A second PCR amplification was performed to incorporate Illumina adapters and barcodes: a first denaturation a at 94°C (1 minute), followed by 12 cycles of denaturation at 94°C (60 seconds),

primer annealing at 55°C (60 secondes) and extension at 68°C (60 seconds) with a final elongation at 68°C for 10 minutes. Amplicons were purified with a ratio at 0.7 of magnetic beads and quantified with the Quant-iTTM PicoGreen® dsDNA Assay Kit (Invitrogen). All the amplicons were pooled in equimolar concentrations and the concentration of the equimolar pool was monitored with quantitative PCR (KAPA SYBR® FAST, Merck). Amplicons libraries were mixed with 10% PhiX and sequences with a MiSeq reagent kit v3 600 cycles (Illumina).

Sequence data processing

Sequencing fastq files were processed in Qiime2 after primer removal (Qiime2 version: 2022.8.0)[53] . Sample fastqs were imported to Qiime2 format and demultiplexed, if necessary, with the qiime demux emp-paired protocol. Samples were denoised, truncated and merged at 100% sequence identity using Dada2 in Qiime2, with the truncation lengths found in supplemental Table S1.1[54]. 16S rRNA gene taxonomy was assigned with the Silva database release 132 for French datasets, and release 138 for Michigan datasets, and taxonomy and ASV tables were exported for further analysis in R[55].

Analysis

Data analyses were performed in R version 4.3.1 and R Studio version 2023.06.1+524[56]. ASV, taxonomy and metadata tables were imported into the phyloseq package 1.44.0[57]. Sequences derived from 16S rRNA genes that were unclassified at the phylum-level, affiliated to Chloroplasts and Mitochondria were removed. The identification of sequence contaminants was assessed with decontam v 1.20.0[58] using the prevalence of ASVs in samples and negative controls (threshold employed listed in Table S1.1).

Rarefaction curves for each dataset were made using the rarecurve() command in the vegan package version 2.6-4[59] and datasets were rarefied using the phyloseq rarefy_even_depth() command (rarefaction levels listed in Table S1.1). Datasets were then subset for further analysis using the ps_filter() command in the microViz package version 0.10.10[60].

Alpha diversity was assessed using estimate_richness() in phyloseq and a T-test or ANOVA with Tukey Honest Significant Difference post-hoc test where applicable. Alpha diversity figures were created using the plot_richness() command from phyloseq with the ggplot2 package version 3.4.2[61]. Bray-Curtis distances were calculated with vegdist(), and PERMANOVA statistical tests were performed with adonis2(), both from the vegan package. Post-hoc analysis on the PERMANOVA results was performed with pairwise.adonis2() from pairwiseAdonis version 0.4.1[62]. Beta diversity ordinations were created with ordinate() from phyloseq with ggplot2. Beta dispersion was assessed with betadisper() and permutest() from the vegan package with the spatial median method[63]. Indicator species analysis was performed with indicspecies package version 1.7.14[64,65] and Venn Diagrams were produced with the VennDiagram package version 1.7.3[66]. Plant health data was analyzed with T-Tests or ANOVA with TukeyHSD post-hoc tests where applicable, and figures made with ggplot2. Additional data wrangling and statistics were performed in the tidyverse package version 2.0.0[67] and R stats package, and figures panels were assembled with patchwork version 1.1.3[68].

Data Availability

Data analysis code can be found at (https://github.com/ShadeLab/Drought_multigeneration_study_common_bean). Raw

sequence data for the French samples can be found on the European Nucleotide Archive under accession number PRJEB65346. Raw sequences from the Michigan samples can be found on the NCBI Sequence Read Archive under BioProject accession number PRJNA1058980.

RESULTS

Plant phenotypic traits respond differently to drought stress in different locations

The plant biomass, yield and photosynthetic traits after drought treatment were analyzed in order to assess the impact of the stress treatment. For plants grown in Pays de la Loire, France, with a slightly longer stress period, both bean genotypes (Red Hawk and Flavert) had a significant decrease in the number of pods on the drought-treated plants (Figure 1.1, A.1). However, the decrease in pod number did not result in a significant decrease in seed yield (Figure 1.1, A.2). For Red Hawk beans grown in Michigan, the number of pods was not impacted by drought treatment. However, photosynthetic rate, stomatal conductance and above ground mass were significantly decreased following application of this abiotic stress. These data and trends indicate that the plants were negatively affected by the stress treatments applied in G1.

The plant phenotypic traits after two generations of treatment were analyzed to assess the impact of single-generation or consecutive stress treatments. There was a general impact of the G2 treatment on the plants grown in Pays de la Loire, independent of G1 stress. The number of pods, seeds and above ground biomass were significantly decreased for both genotypes grown in Pays de la Loire (Figure 1.2, A). In addition, the root mass of Red Hawk was significantly increased by drought stress. (Figure 1.2, A.4). For Red Hawk plants grown in Michigan, only the photosynthetic rate and stomatal conductance were significantly altered following drought stress, similar to what was observed during the first stress treatment (Figure

1.2, B.2). These data indicate that the Flavert and Red Hawk plants in Pays de la Loire were greatly impacted by the drought treatment in G2. The Red Hawk plants in Michigan were affected by the drought immediately following treatment, but did not have any significant decrease in biomass as a result of the drought compared to control. The legacy of G1 treatment did not have a significant impact on most plant outcomes in G2, except for Flavert biomass in Pays de la Loire. Flavert plants that received drought treatment in both generations had significantly lower above-ground biomass than plants that were not droughted in G1 (Figure 1.2, A.3). Overall, one generation of stress had a similar impact on the plants as two generations of stress in Red Hawk, but prolonged drought exposure over multiple generations was more detrimental to Flavert plants.

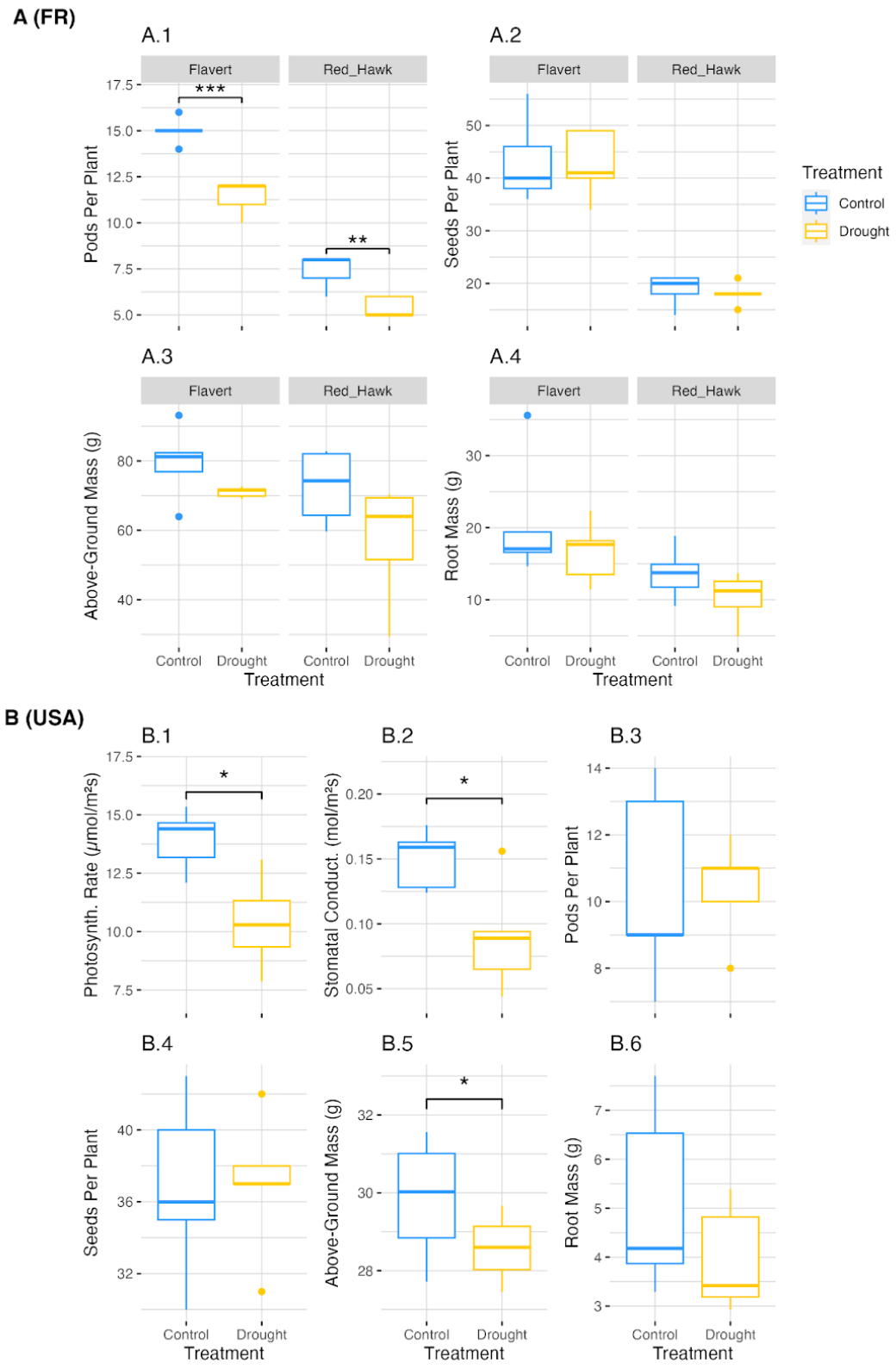

Figure 1.1. Plant phenotypic measurements in Generation 1. **A.** Phenotypic measurements taken for common bean plants of both Flavert and Red Hawk genotypes grown in Pays de la Loire, France. Above-ground and root biomass measurements were taken on fresh plant tissue.

Figure 1.1 (cont'd)
B. Phenotypic measurements taken for common bean Red Hawk plants grown in Michigan, USA. Above-ground and root biomass measurements were taken on dry plant tissue. All above-ground biomass measurements include the total mass of stems, leaves and pods. Photosynth. Rate = Photosynthetic Rate, Stomatal Conduct. = Stomatal Conductance. Welch Two Sample t-test, * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001, n=5 per treatment.

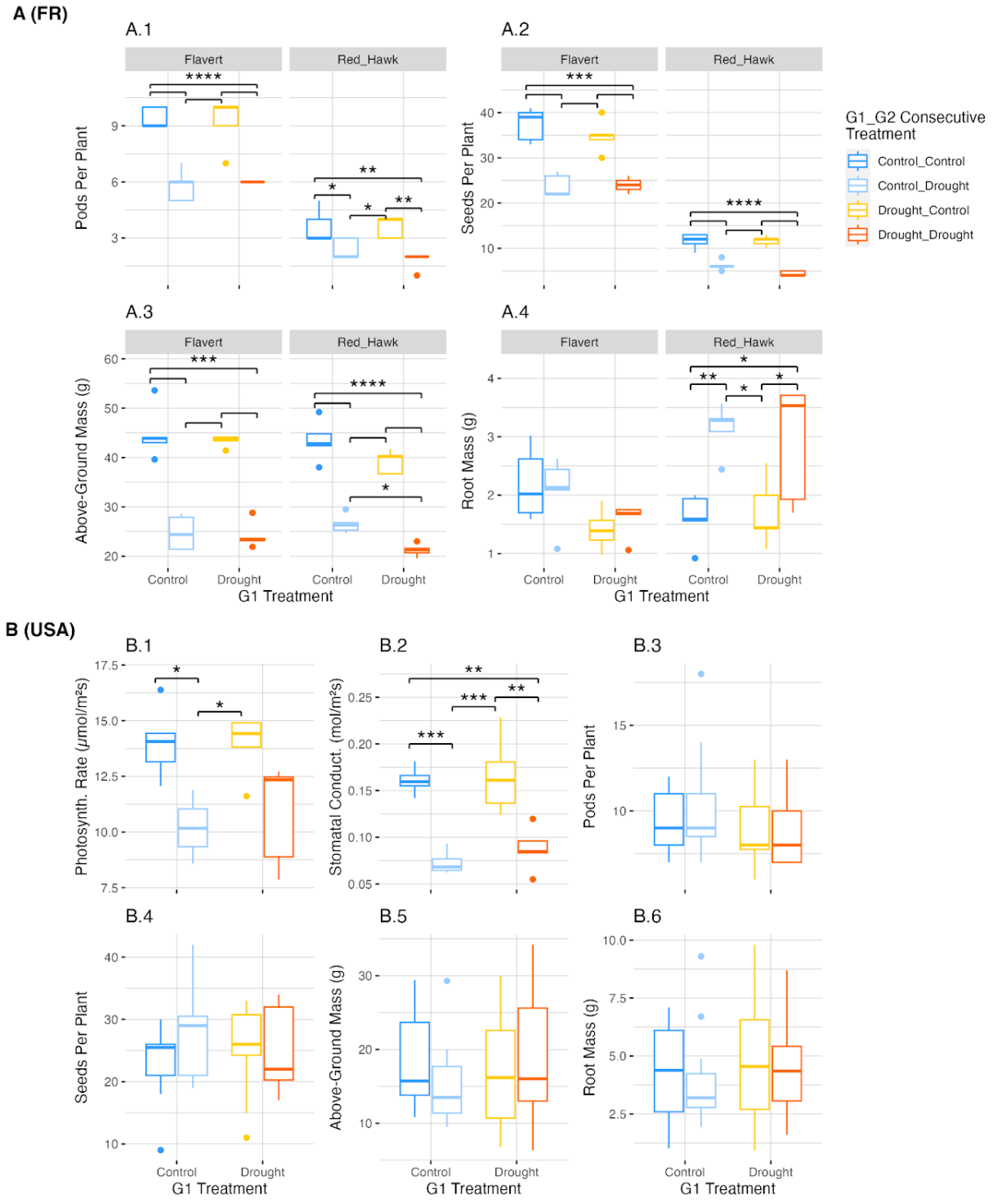

Figure 1.2. Plant phenotypic measurements in Generation 2. **A.** Phenotypic measurements taken for common bean plants of both Flavert and Red Hawk genotypes grown in Pays de la Loire, France. **B.** Phenotypic measurements taken for common bean Red Hawk plants grown in Michigan, USA. Above-ground and root biomass measurements were taken on dry plant tissue. Above-ground biomass measurements include the total mass of stems, leaves and pods.

Figure 1.2 (cont'd)
Photosynth. Rate = Photosynthetic Rate, Stomatal Conduct. = Stomatal Conductance. ANOVA with post-hoc Tukey HSD test, * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001, **** = p-value < 1e-4. Non-annotated significance lines have the same p-value as lines above, n=5 per treatment.

Bacterial richness is affected by drought treatment in Flavert plants

Overall, the bacterial richness observed in samples from Pays de la Loire was much lower than alpha diversity observed in Michigan samples. When sequencing coverage was assessed for the two datasets, both datasets were proficiently sampled and rarefaction curves reach asymptote. When analyzing the datasets separately, significant differences in alpha diversity can be observed between the two genotypes grown in Pays de la Loire in both root and rhizosphere samples in generation 1 (Flavert vs. Red Hawk root samples $p < 0.001$, Flavert vs. Red Hawk rhizosphere samples $p < 0.05$, Figure 1.3A, 1.3C). When assessing the influence of drought treatment on the alpha diversity in G1, most samples were not affected by the treatment, other than a statistically significant increase in alpha diversity in Flavert drought roots compared to the control treatment (Figure 1.3A). There was also an overall trend of increased variability in G1 alpha diversity in the drought treatment across all rhizosphere samples.

In generation 2 alpha diversity, root samples overall were not significantly impacted by the drought treatment in G2 or the legacy of G1 drought (Figure 1.4A, B). There was a trend in increased root alpha diversity in French plants that received drought in G1 and control in G2, but this was not statistically supported (Figure 1.4A). The two genotypes grown in Pays de la Loire were statistically different from each other in the rhizosphere samples (Flavert vs. Red Hawk rhizosphere samples $p < 0.001$, Figure 1.4C). Additionally, Flavert G2 rhizosphere samples

had significantly decreased alpha diversity in plants that received drought in G1 compared to G1 control (Flavert G1 Control vs. Flavert G1 Drought $p < 0.001$, Figure 1.4C). Flavert plants that received drought for the first time in G2 also had significantly decreased alpha diversity compared to Control_Control plants, and higher variability (Figure 1.4C). However, the legacy of G1 drought on the G2 outcome was not reflected in either Red Hawk rhizosphere dataset, and the Red Hawk rhizosphere alpha diversity was not significantly affected by the drought in generation 2 (Figure 1.4C, D). Overall, genotype was found to play a significant role in determining root and rhizosphere alpha diversity, and Flavert plants were the only plants with significantly altered alpha diversity under drought conditions. Red Hawk plants had relatively consistent alpha diversity in both compartments, and despite Red Hawk plant traits being impacted by drought in Pays de la Loire, the alpha diversity of these plants was not affected.

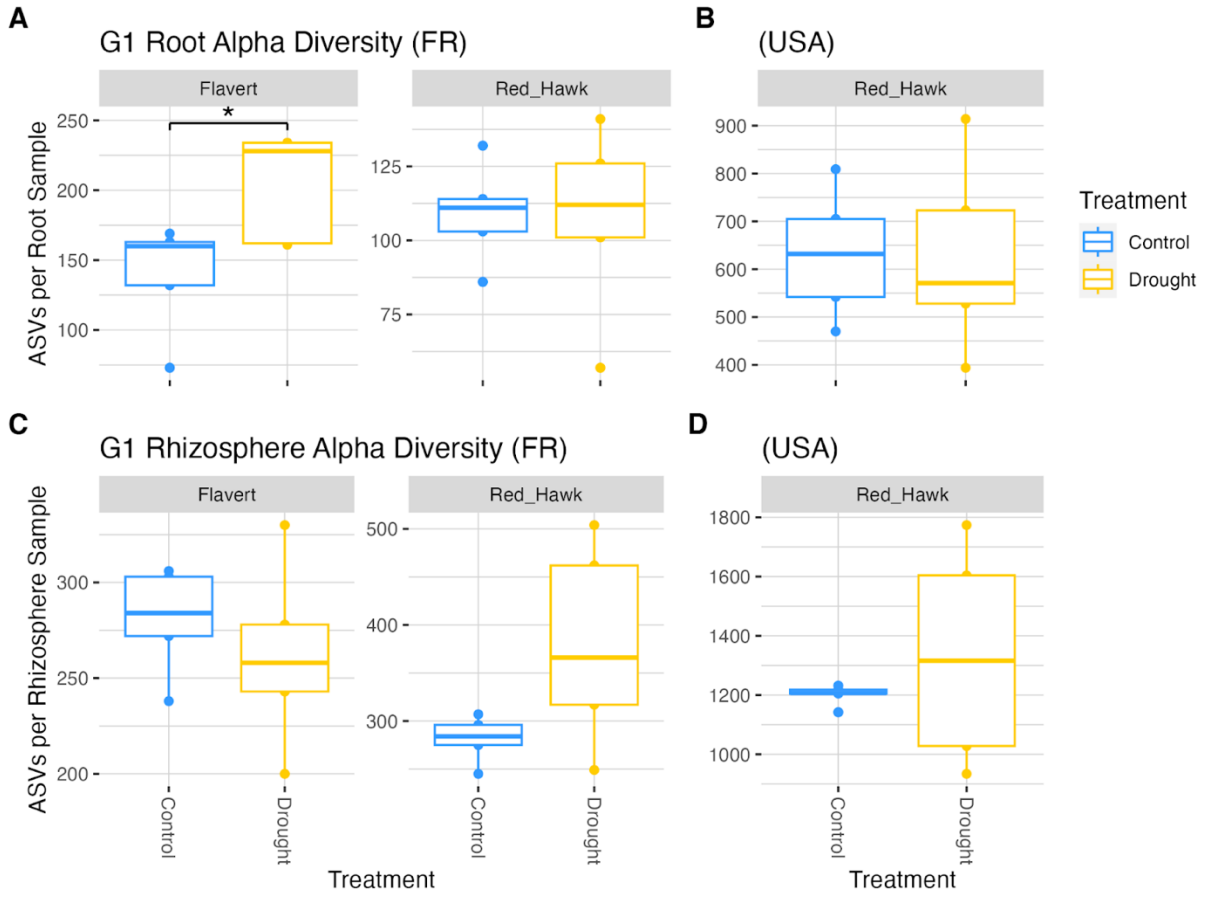

Figure 1.3. Alpha diversity observed for root and rhizosphere samples in Generation 1. **A.** Root samples of plants grown in Pays de la Loire, France. **B.** Root samples of plants grown in Michigan, USA. **C.** Rhizosphere samples of plants grown in France. **D.** Rhizosphere samples of plants grown in USA. Plant genotype is indicated by bars above the panels. Tukey HSD test, * = p-value < 0.05, n=5 per treatment.

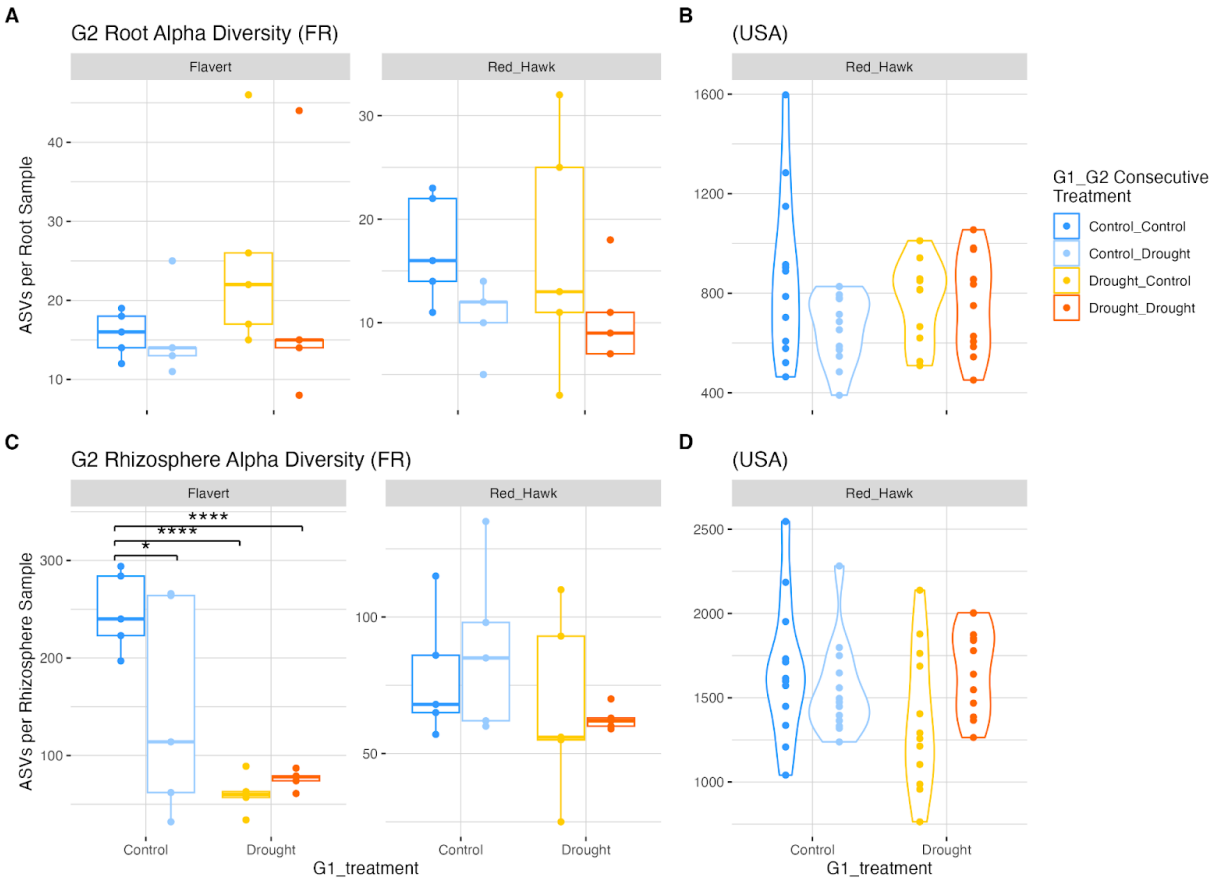

Figure 1.4. Alpha diversity observed for root and rhizosphere samples in Generation 2. **A.** Root samples of plants grown in Pays de la Loire, France. **B.** Root samples of plants grown in Michigan, USA. **C.** Rhizosphere samples of plants grown in France. **D.** Rhizosphere samples of plants grown in USA. Plant genotype is indicated by bars above the panels. ANOVA with Tukey's Honest Significant Difference test, * = p-value < 0.05, **** = p-value < 1e-4. France n=5 per treatment, USA n=12 per treatment.

Drought legacy effects are observed in bacterial community structure

Bray-Curtis dissimilarity was used to assess beta diversity across the root and rhizosphere microbial communities, and was analyzed separately for plants grown in Pays de la Loire and Michigan. In G1, there were significant differences in the bacterial community structure of the two genotypes grown in Pays de la Loire in both root ($p < 0.001$, Figure 1.5A, B) and rhizosphere ($p < 0.05$, Figure 1.5D, E) samples (PERMANOVA). When the genotypes were analyzed separately, there was no influence of the drought treatment in G1 on the root sample

beta diversity in Pays de la Loire, but there were significant differences between drought and control in the rhizosphere communities for both Flavert ($p < 0.01$, Figure 1.5D) and Red Hawk ($p < 0.05$, Figure 1.5E) genotypes (PERMANOVA). The drought treatment did not have a significant effect on the root or rhizosphere communities in Michigan plants in G1 (Figure 1.5C, F).

In G2 there were also significant differences between the two plant genotypes in Pays de la Loire for both compartments (root p-value < 0.001 Figure 1.6A, B, rhizosphere p-value < 0.01 Figure 1.6D, E, PERMANOVA). Flavert roots were significantly affected by the interaction of the G1 – G2 treatments (PERMANOVA, $p < 0.01$, Figure 1.6A), with significantly different communities identified between the Control_Control and Drought_Control plants, which is consistent with alpha diversity results (Pairwise multilevel comparison with pairwiseAdonis in R, $p < 0.01$) and between the Drought_Control and Drought_Drought plants ($p < 0.05$), with variability observed between the Control_Drought and Drought_Drought plants ($p=0.06$). Red Hawk roots in G2 were unaffected by either generation of treatment in Pays de la Loire (Figure 1.6B), but the Michigan Red Hawk plants had significantly different root communities based on the G2 treatment (PERMANOVA, $p < 0.01$, Figure 1.6C). In the G2 rhizosphere samples, Red Hawk plants in Michigan were not affected by either generation of treatment (Figure 1.6F), but in Pays de la Loire, Flavert plants had significantly different communities based on the legacy treatment from G1 ($p < 0.001$, Figure 1.6D, PERMANOVA), and Red Hawk plants were slightly influenced by the treatment in G2 ($p = 0.053$).

These results indicate that the beta diversity of the rhizosphere samples in Pays de la Loire was significantly impacted by the drought treatment in G1 in both genotypes, while Michigan samples were not affected by the drought treatment until the second generation.

Flavert plants were affected by the stress treatment in the second generation in both roots and rhizosphere and presented legacy effects from the G1 treatment on the bacterial communities in G2, while Red Hawk plant communities were only affected by the G2 drought treatment.

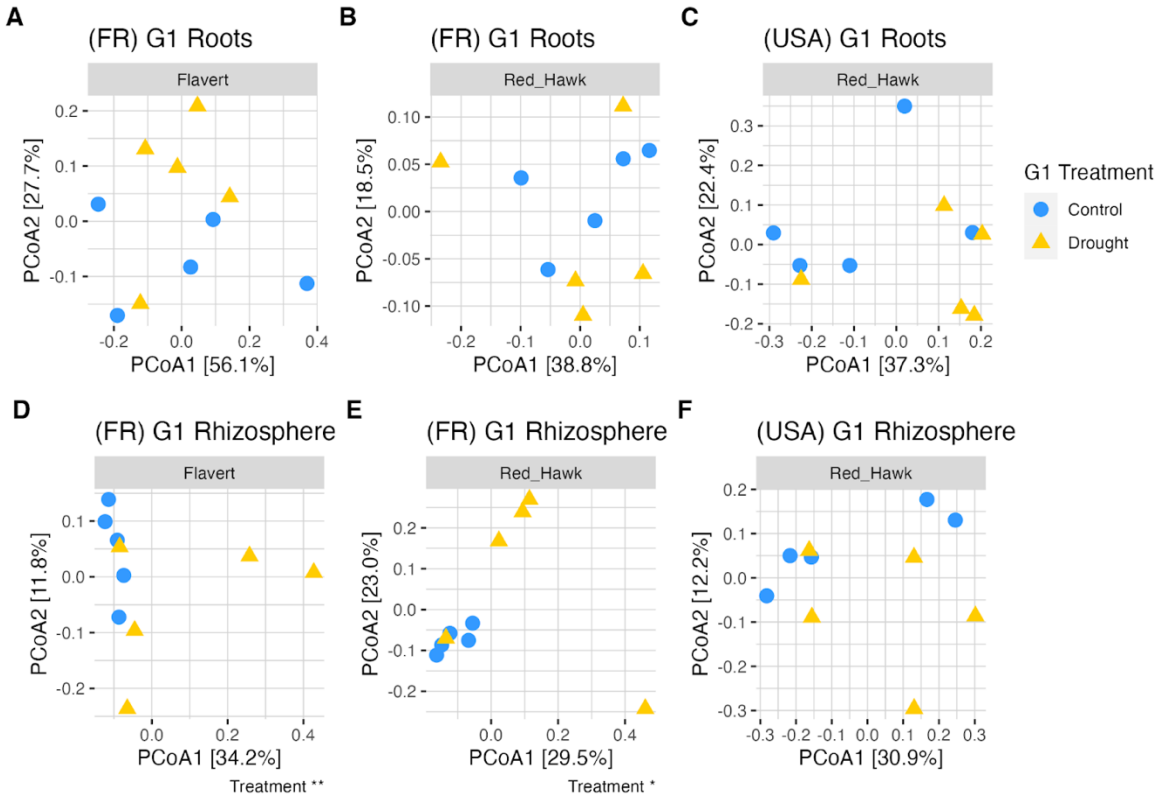

Figure 1.5. PCoA ordinations of beta diversity Bray-Curtis distances in Generation 1. **A, B.** Root samples of Flavert and Red Hawk plants grown in Pays de la Loire, France. **C.** Root samples of plants grown in Michigan, USA. **D, E.** Rhizosphere samples of Flavert and Red Hawk plants grown in Pays de la Loire, Frace. **F.** Rhizosphere samples of plants grown in Michigan, USA. PERMANOVA analysis found significant differences between Genotypes in both root (p-value < 0.001) and rhizosphere (p-value < 0.05) samples in France. Plant genotype is indicated by bars above the panels. * = p-value < 0.05, ** = p-value < 0.01, n=5 per treatment.

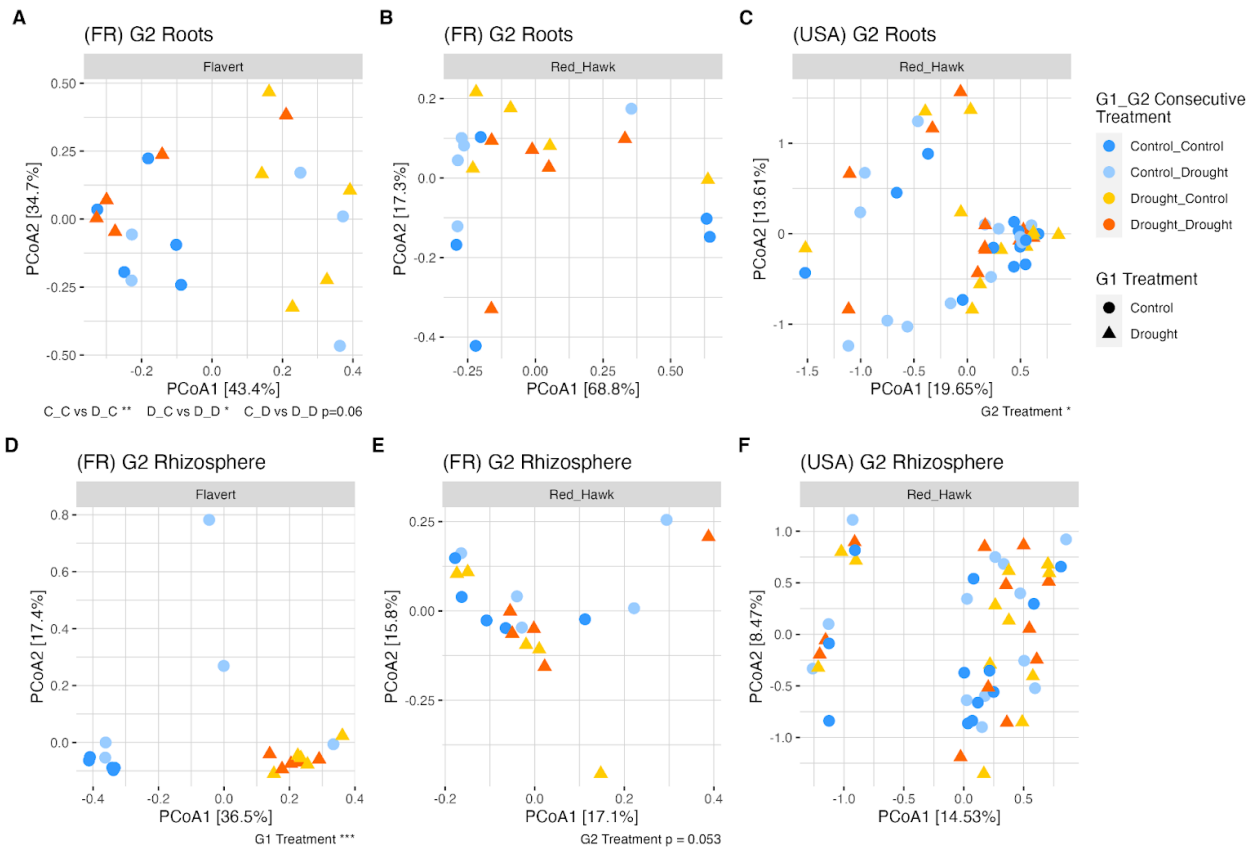

Figure 1.6. PCoA ordinations of beta diversity Bray-Curtis distances in Generation 2. **A, B.** Root samples of Flavert and Red Hawk plants grown in Pays de la Loire, France. **C.** Root samples of plants grown in Michigan, USA. **D, E.** Rhizosphere samples of Flavert and Red Hawk plants grown in France. **F.** Rhizosphere samples of plants grown in the USA. PERMANOVA analysis found significant differences between Genotypes in both root ($p < 0.001$) and rhizosphere ($p < 0.01$) samples in France. Plant genotype is indicated by bars above the panels. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001. France n=5 per treatment, USA n=12 per treatment.

Bacterial community variability is altered by treatments in Generation 2

The dispersion of the Bray-Curtis dissimilarity was assessed using the spatial median between ordination points in order to assess the similarity between samples within treatments. Drought treatment in generation 1 did not have a significant impact on the dispersion of the root and rhizosphere communities (data not shown). In generation 2, Flavert plants in Pays de la Loire had significant changes in community dispersion, with Control_Drought plants having greater dispersion than Control_Control in the root samples (Permutational pairwise analysis, p

< 0.05, Figure 1.7A), and greater dispersion than all other treatment groups in the rhizosphere samples (Tukey HSD, C_D vs. C_C p < 0.001, C_D vs D_C p < 0.05, C_D vs. D_D p < 0.05, Figure 1.7B). Dispersion of communities in Red Hawk G2 plants was unaffected by drought in Pays de la Loire, but Red Hawk plants in Michigan showed a statistically significant decrease in dispersion in the rhizosphere of plants treated with drought stress in G2, regardless of G1 treatment (Permutational pairwise analysis, p < 0.05). Consistent with the effects observed in alpha and beta diversity, the dispersion of the bacterial community in the Control_Drought Flavert plants were significantly altered in G2 indicating legacy effects for these plants, and the Red Hawk rhizosphere community dispersion was affected by the G2 drought in Michigan.

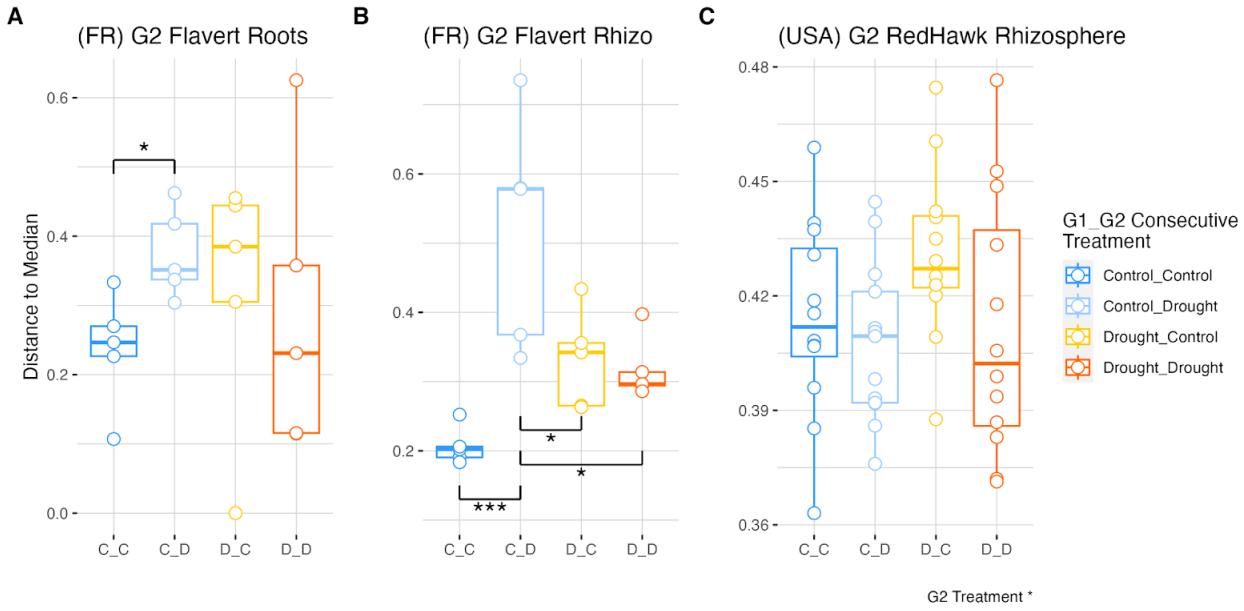

Figure 1.7. Beta dispersion calculated on the spatial median between samples. Only figures showing groups with significance are shown, all other ordinations had no significant differences in dispersion between groups. **A.** Distance to median in Generation 2 Flavert roots in Pays de la Loire, France. **B.** Distance to median in G2 Flavert rhizosphere in France. **C.** Distance to median in G2 Red Hawk rhizosphere in Michigan, USA. Permutational pairwise analysis with Tukey HSD posthoc test, * = p-value < 0.05, *** = p-value < 0.001. France n=5 per treatment, USA n=12 per treatment.

Indicator taxa are associated with drought treatment

Indicator taxa were analyzed to identify ASVs that were significantly associated with the treatment groups and compartments within each generation. A greater number of indicator taxa were found in the Red Hawk plants grown in Michigan than the plants grown in Pays de la Loire, which aligns with the higher overall species richness in the Michigan plants (Table 1.1). In generation 1, there were indicator taxa identified between the control and drought treatment for all plant genotypes and compartments, while generation 2 plants in Pays de la Loire had fewer distinct groups of indicator taxa (Table 1.1). For plants grown in Michigan, indicator taxa could be associated with all G1_G2 treatment groups, as well as the control or drought treatment applied in G1 and G2 (Table 1.1, Figure S1.2, S1.3). Plants grown in Pays de la Loire only had indicator taxa associated with some of the treatment groups in either genotype or compartment (Table 1.1).

Table 1.1

Indicator taxa associated with each treatment group. Numbers represent the number of bacterial ASVs identified that are significantly associated with each treatment group or treatment combination for each location, genotype, and compartment.

	Location:	**France**				**USA**	
	Genotype:	**Flavert**		**Red Hawk**		**Red Hawk**	
	Compartment:	**Root**	**Rhizo**	**Root**	**Rhizo**	**Root**	**Rhizo**
Gen 1	Control	0	28	3	1	8	11
	Drought	28	5	3	50	21	10
Gen 2	G2 Control	1	17	0	3	112	53
	G2 Drought	2	0	0	2	43	67
	Control_Control	0	63	0	1	42	32
	Control_Drought	0	3	0	2	2	17
	Drought_Control	1	0	0	0	62	23
	Drought_Drought	1	0	0	1	18	43
	Control in G1	0	120	0	5	12	83
	Drought in G1	0	0	0	0	48	49

There was no overlap of specific indicator taxa ASVs between the two geographic locations, so ASVs were identified to the taxonomic order level and the overlap of orders that were associated with the drought treatment across generations was assessed. For Red Hawk plants grown in Michigan, there were 3 orders identified that were associated with the root samples in G1 drought, G2 overall drought, and also G2 Drought_Drought plants, specifically. These orders were Xanthomonadales, Pseudonocardiales, and Sphingomonadales. Additionally, there was one order found as indicator taxa in all three drought groups in the rhizosphere, Rhizobiales (Figure 1.8). In Flavert plants grown in Pays de la Loire, there was one order identified as indicator taxa in the root samples in all three groups, Pseudomonadales, and between the G1 drought and overall G2 drought roots, Streptomycetales. Flavert rhizosphere samples did not have any indicator taxa in the G2 drought treatment, but did have 4 orders identified in G1 drought: Xanthomonadales, Cytophagales, Micrococcales, and Salinisphaerales

(Figure 1.8). Red Hawk plants grown in Pays de la Loire had 2 orders identified in the rhizosphere that overlapped between G1 drought and G2 overall drought, Xanthomonadales and Rhizobiales, and one order overlapping between G1 and the G2 Drought_Drought plants identified in the phylum Acidobacteria. French Red Hawk plants did not have any indicator taxa in the generation 2 root samples, but 2 orders, Bacillales and Xanthomonadales, were associated with the G1 drought plants (Figure 1.8). Overlap of taxonomic orders significantly associated with the drought treatment can be identified in each plant genotype and compartment, as well as across the two genotypes and experimental locations. This provides evidence that these bacterial orders may play an important role in the plant microbiome under drought.

Specifically at the ASV level, there were a small number of ASVs that were significantly associated with the drought treatment that overlapped between both generations in the Michigan root samples and between compartments in the Michigan rhizosphere samples (Table 1.2). There were two ASVs identified in the Michigan root samples that were associated with both the G1 and G2 drought treatments, both from the genus *Lysobacter*. Additionally, two ASVs were identified that were associated with both the root and rhizosphere drought treatments in the Michigan G2 plants, from the genera *Promicromonospora* and *Fluviicola*. The overlap of specific ASVs across generations and compartments may suggest that these specific taxa are important for the Red Hawk plants under drought stress in Michigan.

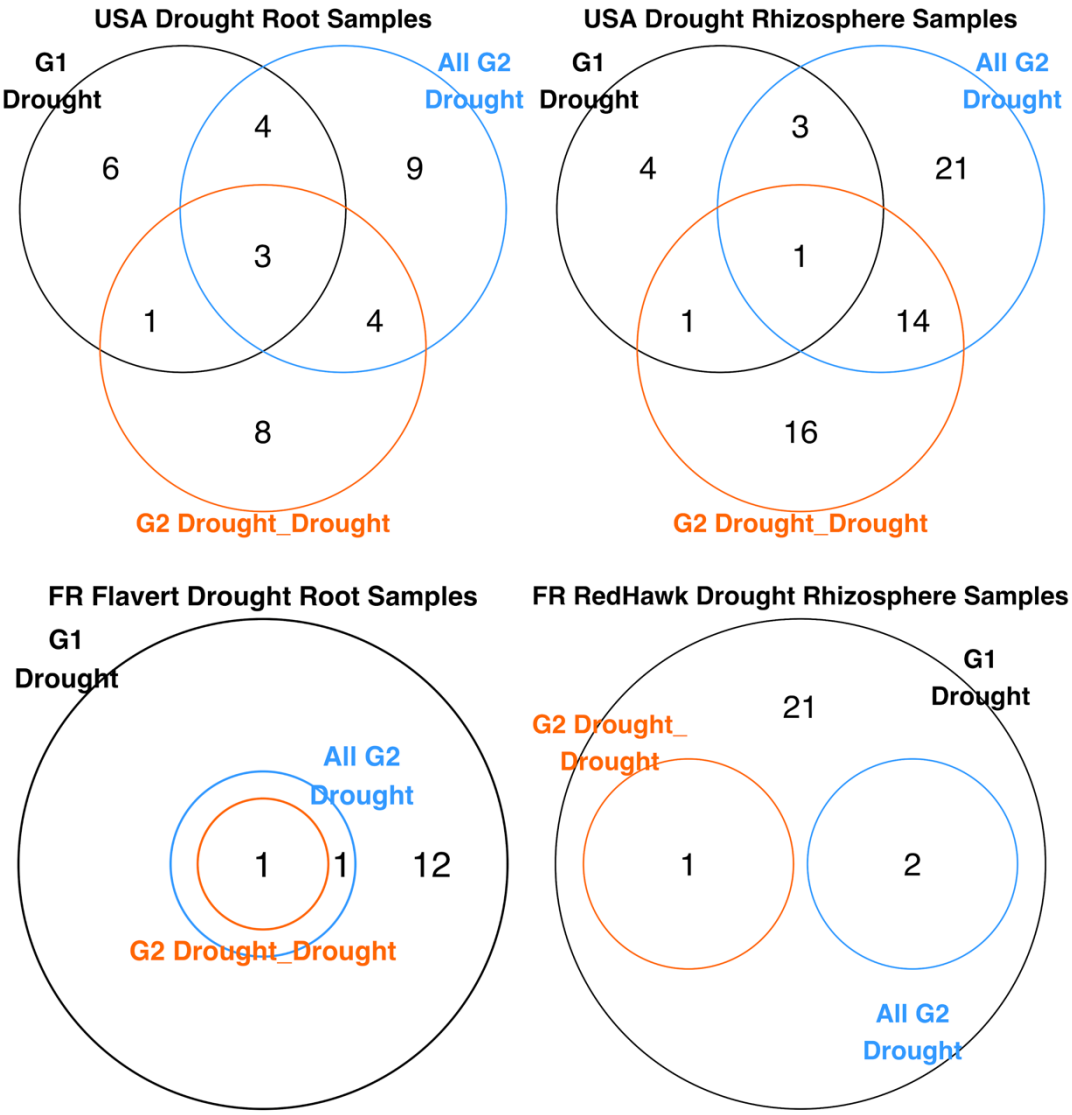

Figure 1.8. Number of indicator taxa at the taxonomic order level that are associated with G1 Drought plants, all G2 Drought plants, and Drought_Drought plants in G2, in each location, genotype and compartment. Flavert plants in France did not have any significant indicator taxa in the G2 Drought treatments in the rhizosphere. There were 4 taxonomic orders identified in the Flavert G1 Drought Rhizosphere. Red Hawk plants in France also did not have any significant indicator taxa in the G2 Drought treatments in the root samples. There were 2 taxonomic orders identified in the Red Hawk G1 Drought Roots.

Table 1.2

Indicator taxa ASVs that were found overlapping between generations or compartments in Michigan drought treatments.

ASV ID (Silva)	Association	Phylum	Class	Order	Family	Genus
3a66a38841ba6877568198b9dac3a1dc	G1 and G2 drought, roots	Proteo-bacteria	Gamma-proteobacteria	Xantho-monadales	Xanthomonadaceae	Lysobacter
f5c494fd1da6eac3404c69c863c35c07	G1 and G2 drought, roots	Proteo-bacteria	Gamma-proteobacteria	Xantho-monadales	Xanthomonadaceae	Lysobacter
47ee012e6bf01ba25c1e9d73e2241846	G2 drought, roots and rhizo	Actino-bacteriota	Actinobacteria	Micrococcales	Promicro-monosporaceae	Promicro-monospora
7e2b7a47670a5cb9656744205ad8b31f	G2 drought, roots and rhizo	Bacteroidota	Bacteroidia	Flavobacteriales	Crocinitomicaceae	Fluviicola

DISCUSSION

Our study contributes to a significant knowledge gap in investigating the effects of drought on the plant microbiome over consecutive plant generations. Overall, the common bean plants in our experiment were negatively impacted by the drought stress in both generations. The stress did not have as great of an impact on the plants grown in Michigan, which was likely due to the stress period being about 1 week shorter in the experimental design than it was in Pays de la Loire. The Flavert and Red Hawk plants in Pays de la Loire experienced a longer drought period, which had a large effect on plant traits in the second generation. The Red Hawk plants that received drought in both generations had lower above-ground biomass than any other treatment combination. Paired with an increase in root biomass in the generation 2 plants under drought stress, this aligns with other studies that have observed increased root length, depth, and biomass in plants under drought stress[34]. These results support our hypothesis that the plants would be significantly impacted by the drought treatment, and partially support the hypothesis that repeated drought exposure has

consequences for the plant health in the second generation, as only the Red Hawk plants in Pays de la Loire showed evidence of a legacy of G1 drought affecting G2 plant outcomes.

In addition to the impacts of the drought stress on the plant biomass and yield, the microbiome communities of the plant roots and rhizosphere were also significantly altered by the drought treatments in one or more compartments in both generations, which supports our second hypothesis. An intriguing element to our experiment was completing the experiment with two genotypes of common bean to understand the effects of the drought in two cultivars that have different domestication histories[45,69]. The two genotypes grown in Pays de la Loire had significantly different community diversity in the roots and rhizosphere communities, even when grown in the same soil and growth conditions. This is aligned with other evidence in the literature that different plant species harbor distinct core microbiota, and may suggest that microbial treatments in agriculture need to be specifically aligned to the plant genetic background to which they are being applied[42,70,71]. Over the two generations, the bacterial communities of the Red Hawk plants appeared to be more resistant to drought stress compared to the Flavert plants, as the Flavert root and rhizosphere microbiome was significantly altered in both generations and plant compartments. This may be a result of kidney beans being bred for more drought resistance traits than flageolet beans throughout their domestication history, leading to reduced stress signaling from the plants under drought which may recruit a more resistant microbiome as well[72–74].

While we can identify significant effects of the drought treatment on the microbiomes in both generations, the root and rhizosphere microbiomes are also highly variable between plants, and greater replication in our study may have allowed for more significant trends in the

data. Despite this, there is a clear effect of the legacy of G1 drought on the microbiome of the Flavert plants in generation 2, and this is also reflected in the plant trait data. When designing our experiment, we predicted that there could be beneficial plant microbiome members enriched in the first generation of drought, that could be passed on to the following generation via vertical transmission, thus resulting in better health outcomes in repeated stress when primed with a helpful microbiome from parental plants[35,43,75–79]. This is not evident in our experiment, as the G2 Flavert plants that received drought in both generations fared the worst in plant health outcomes, and plants that received control treatment followed by a drought had significant shifts in their rhizosphere community structure and dispersion. However, these results still support the hypothesis that repeated drought exposure would have consequences for plant health in the second generation.

While the consequences to plant health under repeated drought exposure were negative, it is intriguing that when indicator taxa are analyzed for significant associations with the drought treatment, we can identify taxonomic orders that are consistently enriched in the drought treatment across generations, and even in plants grown in different soils and between the two different genotypes. The taxonomic orders Xanthomonadales and Rhizobiales were strongly associated with the drought-treated plants in the Red Hawk roots and rhizosphere, and Flavert rhizosphere. Both orders contain many taxa that have been identified as having beneficial impacts on plant health, such as *Xanthomonas sacchari*, *Rhizobium calliandrae*, *Rhizobium phaseoli,* prevalent in common bean root nodules, and *Stenotrophomonas rhizophila*, which has been identified as a core seed microbiome member in common bean[38,79–82]. These orders are also known to contain some plant pathogen species such as *Xanthomonas*

citri[83]. Additionally, in the Michigan Red Hawk plants there were specific ASVs that were consistently associated with the drought treatment from the genera *Lysobacter*, *Promicromonospora* and *Fluviicola,* which could be investigated further to understand their role in the plant microbiome under drought stress. The taxa identified in this study may provide important insight into how the root-associated microbiome is supporting the plant during repeated drought exposure, and further investigation is necessary to understand the specific bacterial strains associated with the drought stress and their role in plant health.

In summary, our study sheds light on how repeated exposure to abiotic stress over multiple generations can impact the plant microbiome, and the importance of understanding the impacts of climate change on microbial communities. Alpha and beta diversity of the root and rhizosphere communities were significantly altered by drought stress in both common bean genotypes, with greater effects in the Flavert genotype. It was apparent that the plant microbiomes in the two locations also had a significantly different alpha diversity in both the root and rhizosphere communities, which may have influenced how the microbial communities responded to the drought treatment. Many variables could have been attributed to these differences, including the soil source region and type, the slight differences in stress treatment period, and the different DNA extraction kits used in the two locations. However, trends in the bacterial communities' responses to the drought treatment were still evident despite these differences. Soil type may have played a role in how the Red Hawk plants responded to the drought treatment in the two locations, and future studies could incorporate soils from different regions when conducting plant microbiome growth experiments to address these potential differences.

Drought in agricultural areas is becoming more and more prevalent around the world, and is particularly devastating to developing countries that rely on staple food crops like common bean. It is important that we find sustainable solutions to support agriculture under changing climate conditions, and applications of beneficial microbes may hold great promise for improving our agricultural systems[84–86]. This study provides evidence that repeated seasons of drought stress will likely be detrimental to agricultural crops and their microbiomes over time as climate change continues to affect agricultural regions. The microbial taxa identified here present a potential area of investigation into bacterial taxa that could be harnessed as biological products to support beans, or agricultural crops more broadly, and future research in this area may allow for improved crop production under drought stress in the future.

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APPENDIX A:
CHAPTER 2 SUPPLEMENTARY MATERIALS

Table S1.1

Parameters used in 16S sequence processing.

Dataset	Dada2 trim parameters	Taxonomy assignment	Decontam parameters	Rarefaction
MSU Roots 16S	--p-trunc-len-f 114 \ --p-trunc-len-r 161 \	silva-138-99-515-806-nb-classifier.qza	Threshold 0.1	20,976
MSU Rhizosphere 16S	--p-trunc-len-f 103 \ --p-trunc-len-r 172 \	silva-138-99-515-806-nb-classifier.qza	Threshold 0.1	20,976
INRAE Root and Rhizosphere 16S - Generation 1	--p-trunc-len-f 200 \ --p-trunc-len-r 180- \	Silva 132 99%	Threshold 0.2	
INRAE Root and Rhizosphere 16S - Generation 2	--p-trunc-len-f 200 \ --p-trunc-len-r 130- \	Silva 132 99%	Threshold 0.2	

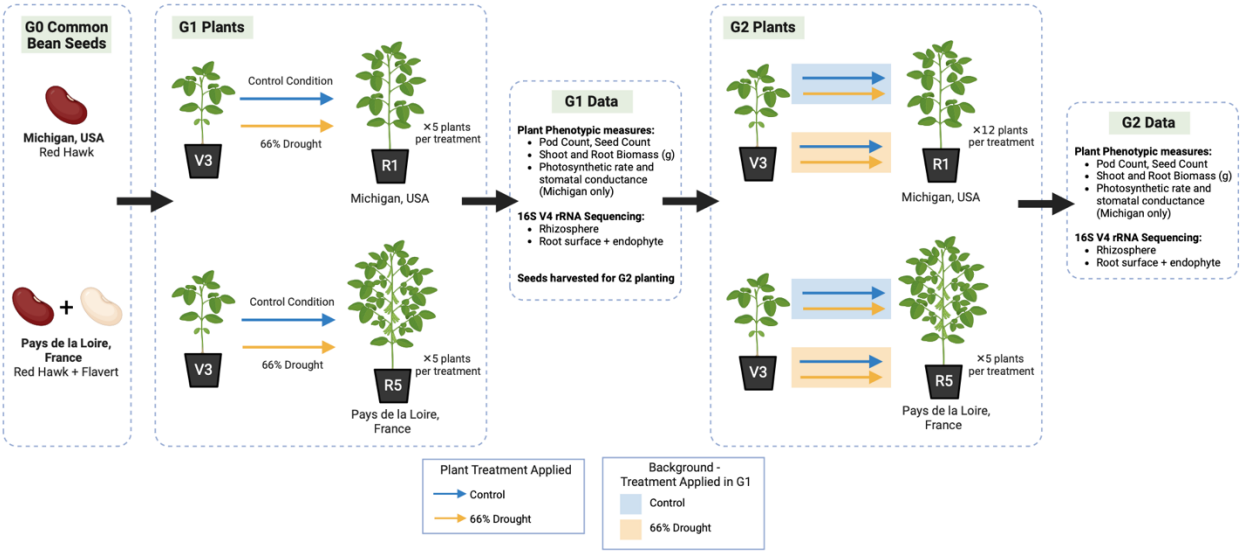

Figure S1.1. Experimental design. *Phaseolus vulgaris L.* (common bean) plants were grown over two generations in a factorial design under either control treatment with ample water and nutrients, or drought treatment with 66% less water and equal nutrient concentration to control plants. The common bean variety "Red Hawk" was grown in Michigan, and both "Red Hawk" and "Flavert" were both grown in Pays de la Loire. Drought treatments were started at the V3 growth stage when plants had three trifoliate leaves expanded, and were concluded at the R1 stage (first open flowers) in Michigan, and the R5 stage (half of the pods filled with discernable seeds) in Pays de la Loire.

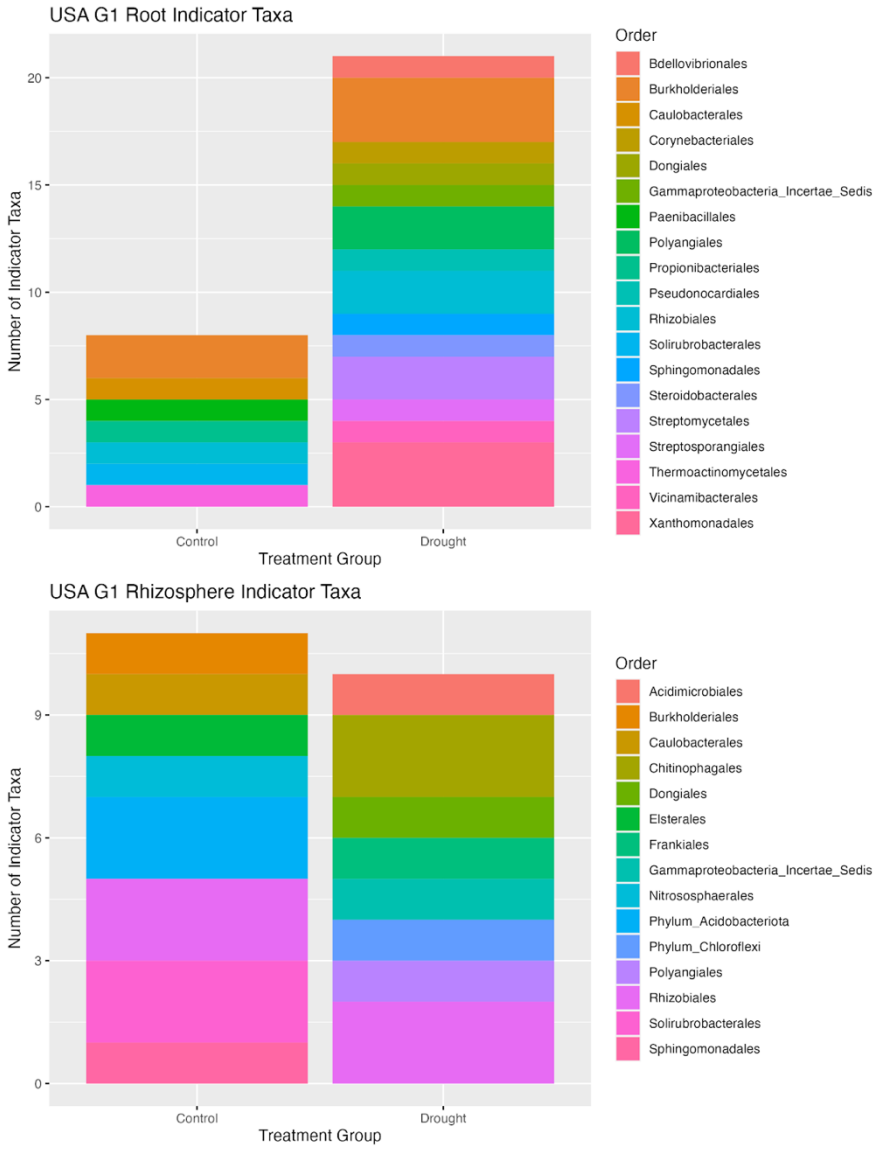

Figure S1.2. Indicator taxa identified in roots and rhizosphere of Generation 1 plants grown in Michigan, USA, grouped by taxonomic order.

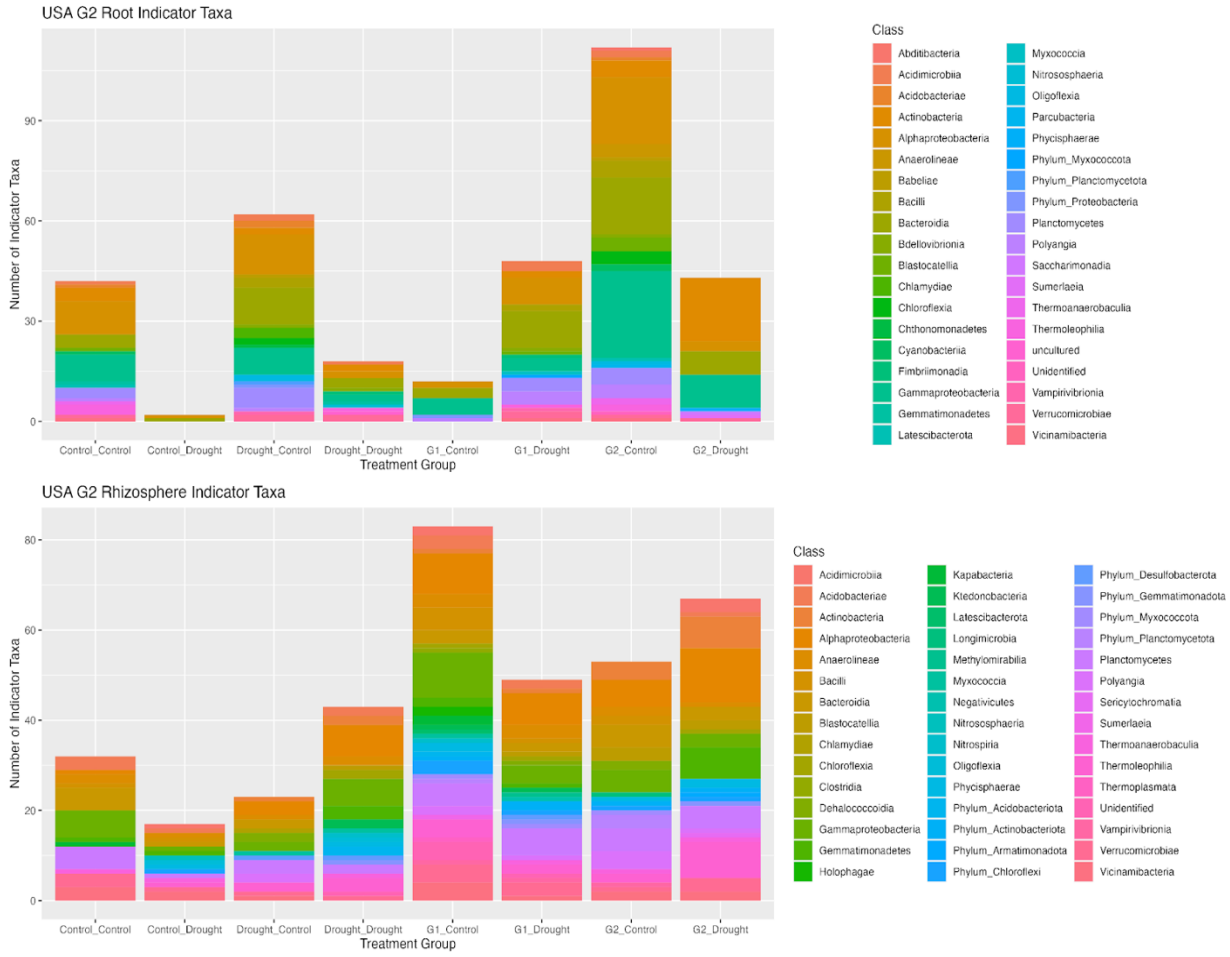

Figure S1.3. Indicator taxa identified in roots and rhizosphere of Generation 2 plants grown in Michigan, USA, grouped by taxonomic class.

CHAPTER 3:

Stable transmission of common bean seed microbiome members over three plant generations despite exposure to abiotic stress

ABSTRACT

The seed microbiome plays an important role in plant microbiome assembly, serving as the starting point for microbiome members to establish in the germinating seedling. Although there is a broad area of work being done on the plant microbiome and its role in supporting plants under biotic and abiotic stress conditions, few studies have addressed the impact of plant stress on the resulting seed microbiome that is passed to the next generation. We conducted a multigenerational study to investigate the impact of drought stress or excess nutrients on the endophytic bacterial community in common bean seeds (*Phaseolus vulgaris L.* var. Red Hawk) while tracking specific parental lines through two generations. The stress treatments had a negligible effect on the resulting seed microbiomes. However, we identified a significant impact of parental line in the offspring seeds and discovered a signature of stable transmission of 22 prevalent seed microbiome members, regardless of plant treatment. These prevalent taxa included previously identified core taxa for common bean, and could be a valuable point of interest for the development of beneficial bacterial applications in agriculture. This study provides insights into the importance of parental line in seed microbiome studies and the maintenance of the plant microbiome across generations, even under challenging environmental conditions.

INTRODUCTION

The plant microbiome is comprised of bacterial, archaeal, and fungal cells that inhabit all plant compartments, including the plant's surfaces, internal tissues, and root structures[1,2]. These microbial species play an important role in plant health through water and nutrient assimilation, conferral of stress tolerance, pathogen defense, and other services[2–4]. Many studies have investigated the unique microbiomes of various plant species, either wild or domesticated. There is an extensive collection of literature on the beneficial rhizosphere, phyllosphere and endosphere microbiome in various plant species, which continue to be growing areas of research as the need for sustainable solutions to improve crop health increases[5–12]. One area of particular interest is the seed microbiome and its role in both pathogen transmission and shaping the beneficial microbiome of the plant[13–15]. While plant microbiomes have been well-documented, few studies have investigated the inheritance of microbiome members over plant generations, especially when mediated through seed transmission[16–22].

Plant microbiomes can be assembled via both horizontal transmission from the environment, and vertical transmission from the parent plant through the seed microbiome[23–28]. Microbial cells can move through the parent plant via vascular tissue or floral compartments and become packaged within the internal seed tissues, and can also colonize seed surfaces[28–32]. When the seed is germinating, priority effects may allow the seed microbiome members to establish first in the microbiome of the new plant, potentially affecting the trajectory of the plant's microbial community[33–35]. Additionally, seed-derived bacteria and fungi have been identified in seedling roots, shoots and rhizosphere compartments in a variety of plant

species[34]. These “inherited” microbiome members enriched by the parent plant may provide important benefits for the offspring plants in the next generation[16,17,36–39].

While the recruitment mechanisms of the seed microbiome have been well documented, only a handful of studies have investigated if the environmental conditions of the parent play a role in determining the microbiome of the resulting seeds[14,40–42]. Parental plant line effects on the seed microbiome have been investigated in a limited number of studies[19,43]. Bintarti et al. found that in common bean plants exposed to drought or provided extra nutrients in environmental growth chambers, differences in the bacterial community of the seeds could be identified based on the treatment that the parent plant received[44]. However, this study had low replication and the soil used was not representative of a true field system. Additionally, fluctuations in wheat seed fungal endophytes have been reported under drought stress conditions, as well as significant shifts in seed bacterial endophytes in rice under salt-stress and *Brassica* in reduced nitrogen environments[45–47]. Consequently, if the seed microbiome can be altered by environmental conditions, then the altered microbiome members that are establishing in the next plant generation may have consequences, either positive or negative, for the next plant’s ability to survive in similar conditions[48]. However, because the seed microbiome has been reported to have low diversity and small community size (tens to hundreds of taxa) combined with high compositional variability for many plant species, a stable signature of vertically inherited taxa may not be expected[18,49,50]. Instead, the seed microbiome may represent an accumulation of stochastically dispersed cells and taxa that are not necessarily plant-supportive, plus some inconsistently detected plant-associated (beneficial or

pathogenic) taxa. Thus, our study aimed to understand how parental plant exposure to abiotic treatments impacted any potential vertical transmission of seed microbiome members.

We conducted a multigenerational experiment in which we exposed common bean plants (*Phaseolus vulgaris L.* var. Red Hawk) to either drought or high nutrients during their early vegetative growth in agricultural soil under controlled growth chamber conditions. We were motivated to study common bean because it is a critical legume for global food security, supporting the health and livelihood of millions of people worldwide[51–53]. The two abiotic treatments were chosen because of their relevance to bean production given the climate crisis. Beans are threatened by drought associated with warming and changes in precipitation patterns[54–56]. Furthermore, beans in the United States and in other areas of high bean production are often managed with excess fertilizer, which can impact the selection and stability of the plant microbiome, such as limiting the diversity of nitrogen-fixing rhizobium found in root nodules[57]. Exogenous nitrogen fertilizers contribute significantly to global greenhouse gas emissions, and it is important to limit any excess use of these products[58].

We hypothesized that (1) Abiotic treatment to the parent plant would characteristically alter the seed endophytic microbiome in alpha and/or beta diversity measures, and (2) Characteristic taxa would be vertically transmitted to the next plant generation that is exposed to the same treatment. Seeds provided from a "Generation 0 (zero)" (G0) seed pool were planted in a growth chamber and exposed to either a control condition with ample water and nutrients, a drought condition with 66% decreased water but equivalent nutrients, or a nutrient condition with 3X concentrated nutrient solution and equivalent water to control. Seeds from these "Generation 1" (G1) plants were harvested and used for growth in "Generation 2" (G2),

where plants were exposed to the treatments in a factorial design, with offspring receiving one of each of the three treatments in G2. 16S V4 rRNA gene amplicon sequencing of the seed bacterial endophyte communities was performed for each of the three generations to assess the impact of the treatments on the seed microbiome and the vertical transmission of the bacterial taxa. This work contributes to a significant knowledge gap in understanding how shifting environmental conditions affect the vertically transmitted seed microbiota over multiple plant generations.

METHODS

Bean cultivar

Phaseolus vulgaris L. var. Red Hawk, developed by the Michigan State University Bean Breeding program was selected as a representative dry bean crop[59] Red Hawk seeds were obtained from the Michigan State University Bean Breeding Program from their 2019 harvest and stored at 4°C until ready for use in experiments. These seeds were obtained as "Generation 0" and used to plant the first generation of the experiment.

Soil preparation

Agricultural field soil was collected for each planting group from the same field location in September 2019, December 2020, May 2021 and September 2021, from a Michigan State University Agronomy Farm field that was growing common bean in 2019 (42°42'57.4"N, 84°27'58.9"W, East Lansing, MI, USA). The soil was a sandy loam with an average pH of 7.2. Five-gallon buckets were used to collect enough soil needed for the planting experiment, avoiding the dry top layer of soil and plant debris. Buckets were covered with air-tight lids and stored at 4°C until the experiment began. Before use in planting, soil was passed through a

4mm sieve to remove rocks and plant debris, and the soil was mixed with autoclaved coarse vermiculite at a 50% v/v ratio for use in growth chamber pots.

Surface sterilization and seed germination

For each planting group, Red Hawk seeds were surface sterilized prior to planting with a solution of 10% bleach and 0.1% Tween20. Seeds were randomly selected from the bulk G0 seed supply, or the harvested G1 seed supply, avoiding seeds that were visibly cracked or moldy. Approximately 20 seeds were placed in sterile 50 mL conical tubes, 20-30 mL of bleach solution was added to the tubes and the seeds were soaked for 10 minutes, with agitation at 5 minutes. After soaking, the seeds were rinsed 5 times with sterile DI H_2O. On the final rinse, approximately 100 μL of rinse water was spread onto Tryptic Soy Agar (TSA) and Potato Dextrose Agar (PDA) plates to assess the efficacy of the seed surface sterilization. TSA plates were incubated overnight at 28°C, and PDA plates were incubated at room temperature for 48 hours. Seeds corresponding to plates with microbial growth were discarded from the experiment and replaced with seeds that were surface-sterile. For germination, seeds were placed in a Petri dish lined with sterile filter paper wetted with 1-2 mL sterile DI H_2O. Petri dishes were stored in the dark at room temperature for 3-4 days, with an additional 2 mL sterile DI H_2O added after 2 days. Once seeds had sprouted radicle roots, they were transferred to soil.

Growth conditions

See Figure S2.1 for experimental design schematic. For G1, three germinated seeds were planted into 3.78 L pots were filled with the soil-vermiculite mixture and placed in a high-light BioChambers FLEX™ LED growth chamber with a 16-hour day/8-hour night cycle at 26°C and

22°C, respectively, and 50% relative humidity. Once seedlings emerged and reached the VC growth stage (vegetative growth with two cotyledons and primary leaves expanded), they were thinned to one seedling per pot. Plants were watered every other day with 300 mL 0.05% 15-10-30 water-soluble fertilizer solution (control condition) (Masterblend International, Morris, IL, USA). At the V3 stage (vegetative growth with third trifoliate leaves expanded), treatments began for the drought- and nutrient-treated plants. Drought plants received 100 mL of 0.15% 15-10-30 fertilizer solution every other day (66% less water than control with the same concentration of nutrients), and nutrient plants received 300 mL of 0.15% 15-10-30 fertilizer solution every other day (3X concentrated nutrients with the same volume of water as control). After approximately 14 days of treatment period, when plants reached the R1 stage (reproductive stage, first open flowers), they were returned to the control-treatment watering regime until senescence. As plants began to dry, they were watered less frequently as needed. Mature seeds were collected from 12 plants per treatment for seed microbiome assessment and for growth for the next generation.

For Generation 2 (G2), seeds from the 36 G1 parental lines that received either control, drought or nutrient conditions were planted in a full factorial design and grown under each of three treatment conditions in G2. There were nine cross-generational treatment combinations total (G1_G2, n=12 plants per treatment combination): Control_Control, Control_Drought, and Control_Nutrient; Drought_Control, Drought_Drought, and Drought_Nutrient; Nutrient_Control, Nutrient_Drought, and Nutrient_Nutrient. Six seeds from each parental line were surface sterilized and germinated as described above, then planted in the field soil-vermiculite mixture in seedling trays in the growth chamber under the conditions stated above.

G2 plants were grown in three randomized planting groups, with each planting group containing parental lines from all three treatments. Once plants reached the VC stage, three healthy seedlings per parent line were transferred to 3.78 L pots. Each G1 parental line provided one offspring per G2 treatment, for a total of 108 plants in G2. Plants were watered according to the conditions and treatment timeline in G1.

Seed harvest

Once the plants had senesced and pods were dried, seeds were harvested for planting or microbiome analysis. Seed pods were removed from each plant and stored in sterile Whirl-pak® bags for transport to the lab. Pods and seeds per plant were counted, and then seeds were removed from the pods and pooled by plant in 50 mL conical tubes and stored at 4°C for use in planting. 5 seeds per plant were selected for microbiome analysis and stored in a separate 15 mL conical tube at -80°C until DNA extraction was performed.

DNA extractions

Seed microbiome DNA extractions were performed on sets of five seeds, which is our unit of microbiome sampling. For the G0 bulk seed, twenty sets of five randomly selected seeds were analyzed from the G0 seed pool. In G1 and G2, seeds were pooled by plant and five seeds per plant were analyzed from the 36 G1 parent plants and 108 G2 offspring plants. Seeds were thawed and surface sterilized according to the method above, and then microbial DNA was extracted from the endophytic compartment using a protocol adapted from Barret *et al.* 2015 and Bintarti *et al.* 2021[24,49]. Following surface sterilization, the seeds were sliced in half lengthwise along the natural division of the cotyledons with a sterile razor blade. Sliced seeds were then placed in a 50 mL conical tube and 20-30 mL of sterile Phosphate-buffered Saline

(PBS) with 0.05% Tween 20 was added. Seeds were soaked overnight at 4°C with constant agitation on an orbital shaker at 160 rpm to allow microbial material to be released from the seed tissues. After soaking, tubes were centrifuged at 4500×g, 4°C, for one hour. Seed tissue and supernatant were removed, and the remaining pellet was transferred to a 1.5 mL microcentrifuge tube. Pellets were stored at -80°C until extraction with the E.Z.N.A. Bacterial DNA kit (Omega Bio-tek, Inc., Norcross, GA, USA) following manufacturer's protocol with the following modifications. To begin the protocol, the seed material pellet was resuspended in 100 μL TE Buffer, 10 μL kit-provided Lysozyme was added, the samples were vortexed thoroughly, and incubated at 37°C for 1 hour. The glass bead step from the E.Z.N.A. kit was utilized with 25-30 mg glass beads, provided, and samples were vortexed at maximum speed for 10 minutes in a 24-tube vortex adapter. After adding the Proteinase K, the samples were incubated in a shaking heat block at 55°C for 2 hours. In the final step, DNA was eluted in 60 μL Elution Buffer and incubated at 65°C for 10 minutes before centrifuging into the final tube.

DNA extractions were performed in randomized batches within each generation (Table S2.1). For each batch, a negative and positive control were included. The negative control was 3 mL sterile PBS+Tween buffer, and the positive control was an aliquot of a mixture of cells from a custom-made mock bacterial community suspended in 3 mL PBS+Tween buffer [60]. These controls were shaken overnight alongside the seed samples and then processed and sequenced as described for the seeds, and then ultimately used to perform batch-informed bioinformatic sequence decontamination [61].

Sequencing

Sequencing of the V4 region of the 16S rRNA gene (515F-806R)[62,63] was performed at

the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory (Lemont, IL, USA). The DNA was PCR amplified with region-specific primers that include sequencer adapter sequences used in the Illumina Nextseq2K flowcell; FWD:GTGYCAGCMGCCGCGGTAA; REV:GGACTACNVGGGTWTCTAAT[62–66]. Each 25 µL PCR reaction contained 9.5 µL of MO BIO PCR Water (Certified DNA-Free), 12.5 µL of QuantaBio's AccuStart II PCR ToughMix (2x concentration, 1x final), 1 µL Golay barcode tagged Forward Primer (5 µM concentration, 200 pM final), 1 µL Reverse Primer (5 µM concentration, 200 pM final), and 1 µL of template DNA. The conditions for PCR were as follows: 94 °C for 3 minutes to denature the DNA, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; with a final extension of 10 min at 72 °C to ensure complete amplification. Amplicons were then quantified using PicoGreen (Invitrogen) and a plate reader (Infinite® 200 PRO, Tecan). Once quantified, volumes of each of the products were pooled into a single tube, in equimolar amounts, this pool was then cleaned up using AMPure XP Beads (Beckman Coulter), and then quantified using a fluorometer (Qubit, Invitrogen). After quantification, the pool was diluted to 2 nM, denatured, and then diluted to a final concentration of 6.75 pM with a 10% PhiX spike for sequencing. Amplicons were sequenced on a 251bp x 12bp x 251bp Nextseq2000 run[65].

Sequence data processing

Sequencing fastq files were processed in QIIME2 after primer removal by the sequencing center (QIIME2 version: 2022.8.0)[67]. Sample fastqs were imported to QIIME2 format, and samples were denoised, truncated and merged using Dada2, with a forward truncation length of 191, and reverse truncation length of 84[68]. Amplicon sequence variants

(ASVs) were defined at 100% sequence identity and 16S taxonomy was assigned with the Silva database release 138, and taxonomy and ASV tables were exported for further analysis in R[69].

Sequence Decontamination

Data analyses were performed in R version 4.3.1 and R Studio version 2023.06.1+524[70]. There were 126.8 million merged DNA reads in the dataset prior to host removal and decontamination. ASV, taxonomy, and metadata tables, and phylogenetic tree files, were imported into the phyloseq package and host reads classified as chloroplast and mitochondria were removed using the subset_taxa() command in the phyloseq package version 1.44.0[71]. 90% of the total DNA reads and 13% of the ASVs were removed as host reads, leaving 12.3 million total bacterial DNA reads. Datasets were decontaminated with the decontam package version 1.20.0 at the 0.1 threshold utilizing the specific negative and positive controls from each extraction group[72]. After decontamination, there were 422,719 total DNA reads with a range of 456-5788 reads per sample in the full dataset. Rarefaction curves were created using the rarecurve() command in the vegan package version 2.6-4[73]. Since seed microbiomes typically have low bacterial diversity containing tens to hundreds of taxa, and vertical transmission of specific ASVs was a primary area of investigation in this study, the full dataset was preserved to ensure full observation ASVs[49].

Analysis

Alpha diversity species richness was assessed using estimate_richness() in phyloseq, and figures were created using the plot_richness() command from phyloseq with the ggplot2 package version 3.4.2[74]. Faith's Phylogenetic Diversity was calculated with calculatePD() from the biomeUtils package version 0.022[75]. ANOVAs were performed with the base R stats

command aov(). Weighted UniFrac distances were calculated with distance() in phyloseq and used for all analyses of beta diversity, and PERMANOVA statistical tests were performed with adonis2() from the vegan package. Post-hoc analysis on the PERMANOVA results was performed with pairwise.adonis2() from pairwiseAdonis version 0.4.1[76]. Beta diversity ordinations were created with ordinate() from phyloseq with ggplot2. Beta dispersion was assessed with the betadisper() and permutest() commands from the vegan package. Datasets were divided into generation or treatment groups where applicable using the ps_filter() command in the microViz package version 0.10.10[77]. Figure 4A was created with the UpSetR package version 1.4.0, and statistical analyses were performed with leveneTest() from the car package and kruskal_test() and dunn_test() from the rstatix package[78–80]. Additional data analysis was performed in the tidyverse package version 2.0.0 and dplyr package version 1.1.2 (Wickham et al. 2019, Wickham et al. 2023). ASV transmission rates were analyzed as count data and Pearson's Chi-squared Tests were performed with chisq.test() from the base R stats package. Seed microbiota were compared to core ASVs identified in Simonin *et al.* 2022[50] and Venn Diagrams were produced with the VennDiagram package version 1.7.3[83]. To compare the taxa in this study to the 48 core bean rhizosphere microbiome taxa identified by Stopnisek and Shade 2021[84], the fasta sequences for each core OTU were used as a query set in a two sequence nucleotide BLAST on the National Center for Biotechnology Information (NCBI) database website, and the fasta sequences from the seed ASVs were compared to the 48 core taxa at >96% identity[85].

Data Availability

Data analysis code can be found at (https://github.com/ShadeLab/Seed_transmission_Common_Bean). Raw sequences can be found on the NCBI Sequence Read Archive under BioProject number PRJNA1058980.

RESULTS

Bean seed microbiomes are stable under environmental treatment in agricultural soil.

The 16S V4 amplicon sequencing of the seed bacterial endophytes was assessed for each of the three generations based on the Weighted Unifrac distance matrix. Since seed microbiomes typically have low bacterial diversity containing tens to hundreds of taxa, and vertical transmission of specific ASVs was a primary area of investigation in this study, the full dataset was preserved[49]. After host removal and decontamination relative to matched DNA extraction controls, there was a range of 456 to 5,788 bacterial DNA reads in the seed samples, which was evidenced to be sufficient to exhaustively observe the low-richness seed microbiomes (Figure S2.2) given the method and its known biases (e.g. DNA extraction kit and sample contamination)[49,61]. Observed ASV richness ranged from approximately 20 to 80 ASVs across all three generations, which is in line with the expected alpha diversity in the samples, and there is no significant influence of treatment on the species richness in either G1 or G2 (ANOVA, G1_treatment: F= 0.150, p-value=0.861. G1_G2: F=0.393, p-value=0.923, Figure 2.1A) (ANVOA, G2 Richness: F=1.122, p-value=0.334, Figure 2.1B), or Faith's Phylogenetic Diversity in G2 (ANOVA, PD: F=1.111, p-value=0.346, Figure 2.1C).

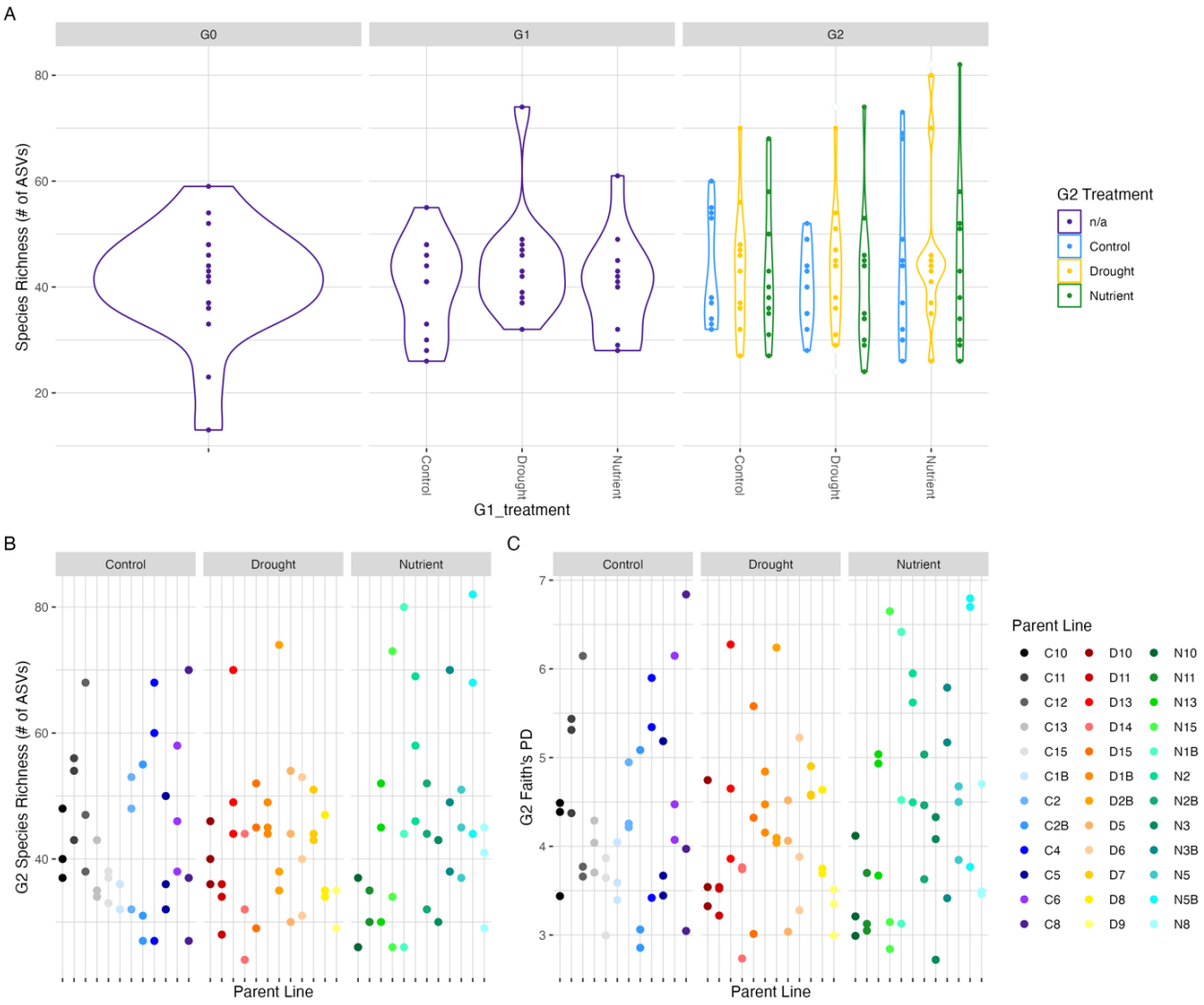

Figure 2.1. Alpha Diversity. **A.** Number of ASVs observed in each of the seed microbiome samples across all generations. Five seeds were used for each sample. G0 n=20 samples, G1 and G2 n=12 samples per treatment group. There is no influence of treatment groups on the species richness observed in either G1 or G2 (ANOVA, G1_treatment: F= 0.150, p-value=0.861. G1_G2: F=0.393, p-value=0.923). **B.** Number of ASVs observed, and **C.** Faith's Phylogenetic Diversity of G2 seed samples by parental line. Gray bars indicate treatment applied to the parent plant in G1. There are no significant differences between parent lines in either Richness or PD measure (ANOVA, Richness: F=1.122, p-value=0.334; PD: F=1.111, p-value=0.346).

When beta diversity of the Weighted Unifrac distance is examined in the full dataset, there is no significant influence of the plant generation on the bacterial communities in the seed samples (PERMANOVA, DF=2, R^2=0.0160, F-value=1.3173, p-value=0.1967, data not shown). Additionally, when the G1 seed microbiomes are analyzed, there is no significant

influence of the plant treatment on the bacterial communities (PERMANOVA, DF=2, R^2=0.06375, F-value=1.1236, p-value=0.2948, data not shown). In generation 2, there was no influence of the legacy of the G1 environmental treatment, or the treatment applied in G2, on the resulting seed microbiome of G2 (PERMANOVA, G1 treatment: DF=2, R^2=0.0194, F-value=1.1149, p-value=0.3038; G2 treatment: DF=2, R^2=0.01504, F-value=0.8646, p-value=0.6496) (Figure 2.2A). These results suggest that with high replication within treatments to account for variation between plants, and growing plants in a representative agricultural soil, the seed microbiome communities are not directly affected by the abiotic treatments applied in this study.

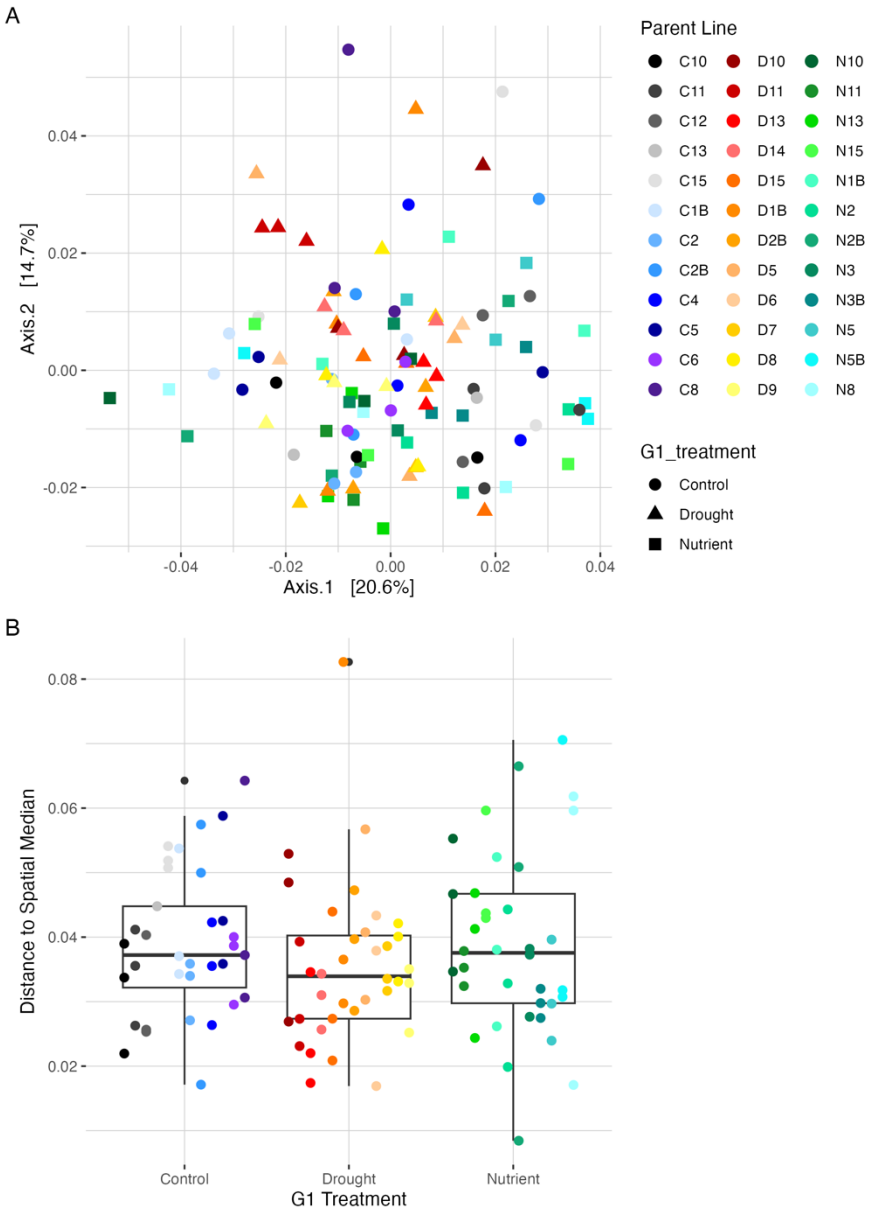

Figure 2.2. Beta diversity in Generation 2 seed samples. **A.** Weighted Unifrac distance of G2 seed samples. Points represent three offspring from each parent line, each of which received a different treatment in G2. Parent plant line is the only significant explanatory variable in the G2 samples (PERMANOVA, r2=0.356, F= 1.2421, p-value=0.0065**). **B.** Beta dispersion around the spatial median of Weighted Unifrac distances in G2 seed samples. Lines are grouped by G1 parent treatment, represented by black boxplots. G1 treatment and parent line are not significant. (ANOVA, G1 Treatment: DF=2, F-value= 1.2835, p-value= 0.2835. Line: DF: 35, F-value=1.0825, p-value=0.3714). Sample G2_9, the line C13 Nutrient offspring, was removed from the figures as an outlier. However, statistics were performed with this sample included.

Parental plant line drives variation in the Generation 2 seed communities.

While there was no influence of the abiotic treatments, the parental plant line that beget the G2 plants was highly significant in explaining the variation in the G2 beta diversity (PERMANOVA: DF= 33, R^2= 0.3568, F-value= 1.12421, p-value=0.0065**) (Figure 2.2A). Additionally, beta dispersion of the Weighted Unifrac distances in the G2 seed communities was consistent between abiotic treatments and parental lines (ANOVA, G1 Treatment: DF=2, F-value= 1.2835, p-value= 0.2835. Line: DF: 35, F-value:1.0825, p-value=0.3714) (Figure 2.2B).

When the G2 samples are grouped by the treatment the G1 parent plants received, the parental lines are not significantly different in control and nutrient lines, while parent line is significant in explaining differences in the drought lines (PERMANOVA, Control: DF=11, R^2=0.3532, F-value=1.2189, p-value=0.0526; Drought: DF=11, R^2= 0.4032, F-value= 1.2353, p-value=0.0021**; Nutrient: DF=11, R^2= 0.3474, F-value= 1.1142, p-value=0.2687) (Figure 2.3A, B, C). These results suggest that the parental line is the most important factor determining the seed microbiome membership in the G2 offspring, and that the differences in community composition between parent lines are increased when the parent plants are exposed to abiotic stress conditions.

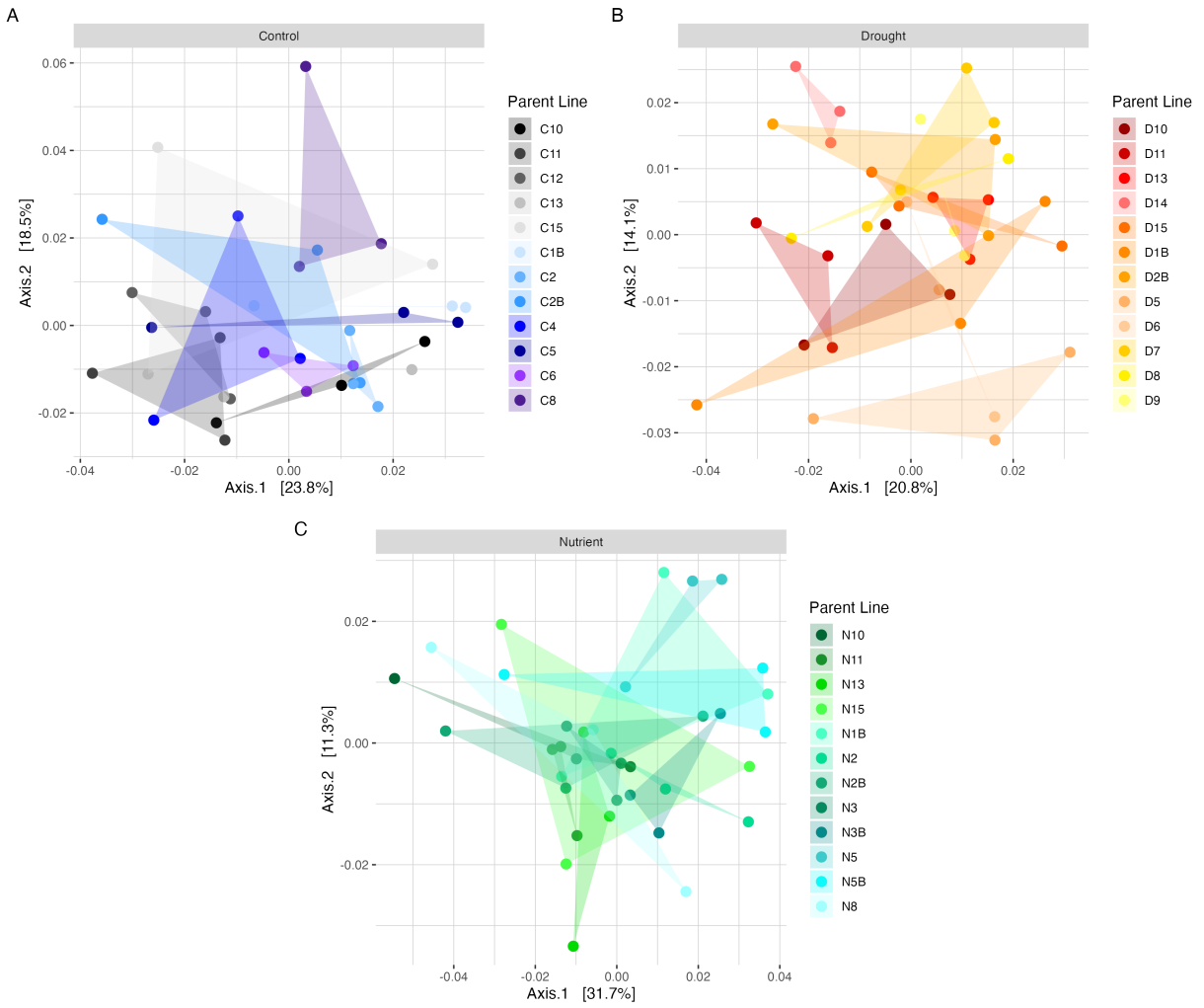

Figure 2.3. PCoA plots of Weighted Unifrac beta diversity in Generation 2, subset by parent plant treatment. When plant lines are grouped by G1 parent treatment, parent plant line is not significant in the control and nutrient lines, but is significant in the drought lines (PERMANOVA, Control: r2= 0.35319, F=1.2189, p=0. 0526, Nutrient: r2= 0.34740, F=1.1142, p= 0.2687, Drought: r2= 0.40311 F=1.4637 p=0.0021**). Bars above the ordinations indicated the treatment applied to the parent plants in G1: **A.** Control, **B.** Drought, **C.** Nutrient. The three offspring from each parent are connected by triangles to aid in visualization. Sample G2_9, the line C13 Nutrient offspring, was removed from figure A as an outlier. However, statistics were performed with this sample included.

Overlapping seed microbiome taxa are present in each parent line and all three generations.

Out of 658 total ASVs detected, there were 128 ASVs shared across all three generations (Figure 2.4A). These ASVs that were detected in all G0, G1 and G2 seeds were in higher relative abundance than the ASVs that were only found in one or two generations (Figure 2.4B)

(Kruskal-Wallis test: test-statistic=363.59, df=2, p-value<0.0001; Post-hoc Dunn's test with Benjamini-Hochberg correction: 1 vs 3 generations: test-statistic=17.78, adjusted p-value<0.0001, 2 vs 3 generations: test-statistic=5.879, adjusted p-value<0.0001). Furthermore, the Genus-level taxonomic profiles of G0, G1, and G2 microbiomes were highly comparable (Figure S2.3), suggesting a consistent taxonomic signature of the ASVs detected across seed generations.

Since vertical transmission is a primary focus of this study, ASVs overlapping between a G1 parent plant and at least one of its offspring in G2, referred to as "overlapping taxa" within lines, were investigated. Overlapping taxa between G1 and G2 can be identified in all 36 parental lines, along with taxa that are unique to one generation (Figure 2.4C). There were 99 taxa identified as overlapping within specific plant lines, 43 of which were common to all G1 treatments (Figure S2.4). The proportion of the ASVs in each line that are overlapping between G1 and G2 range from 17% to 45%, with an average of 29% of ASVs overlapping between parent and offspring in each line (Figure 2.4D).

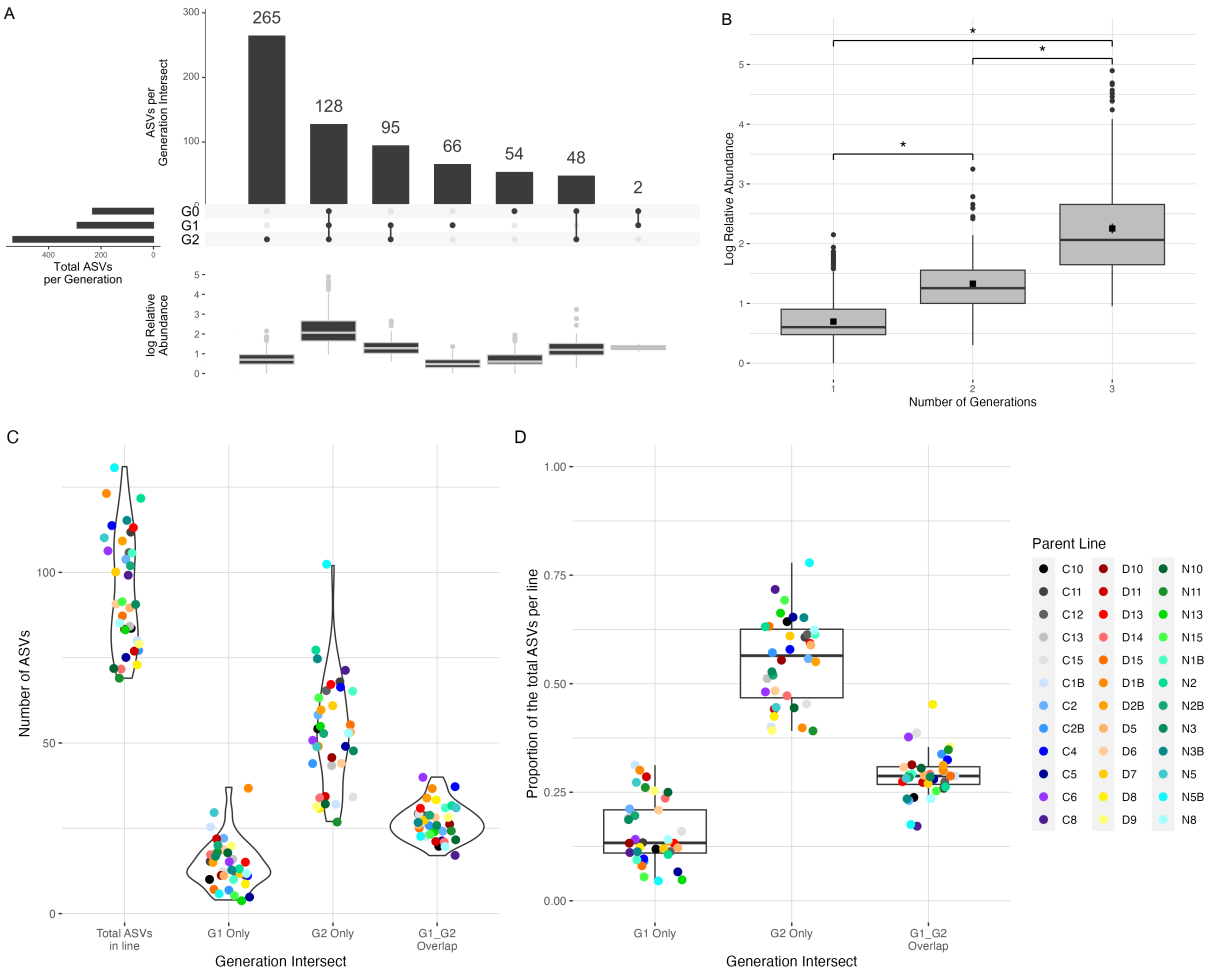

Figure 2.4. Unique and overlapping taxa between generations. **A.** Number of ASVs and relative abundance of ASVs per generation intersect across all samples. G0 n=20 samples (five seeds per sample), G1 n =36, G2 n=108. **B.** Log relative abundance of ASVs based on how many generations in which they are found. Out of 658 total ASVs, taxa found in all three generations are significantly more abundant in the dataset than ASVs found in only one or two generations. Asterisks indicate p-value<0.0001, black squares indicate the mean value. (Kruskal-Wallis test: test-statistic=363.59, df=2, p-value<0.0001; Post-hoc Dunn's test with Benjamini-Hochberg correction, 1 vs 3 generations: test-statistic=17.78, adjusted p-value<0.0001; 2 vs 3 generations: test-statistic=5.879, adjusted p-value<0.0001). **C.** Total number of ASVs per parent line and number of taxa found in G1, G2, or overlapping. "G1_G2 Overlap" is defined as ASVs present in both the G1 sample and at least one G2 offspring within a parent line. 99 ASVs were identified as overlapping within parent lines, and there were overlapping ASVs identified in all 36 lines. **D.** proportion of the total ASVs per line found in G1, G2, or overlapping. Boxplots represent the median values and first and third quartiles, and whiskers represent the 95% confidence interval.

Of the 99 overlapping taxa identified within parental lines, 29 ASVs were found in only one parental line. The 70 ASVs that were found in at least two lines were investigated to understand their prevalence (occupancy in the lines) in the dataset and their rate of transmission in the three offspring in G2 (Figure 2.5, Table S2.2). There were nine ASVs present in all 36 parental lines, and an additional 13 ASVs were found in at least half of parental lines. These 22 prevalent taxa generally had 100% transmission to all three G2 offspring within lines, while less prevalent taxa were not transmitted as consistently in the G2 offspring (Figure 2.5). The transmission of the ASVs to G2 offspring is not significantly impacted by the treatment the G1 parent received, and there is no significant difference in transmission between lines (Pearson's Chi-squared Test. G1 Treatment: $X^2 = 0.67413$, df = 4, p-value = 0.9545. Line: $X^2 = 63.48$, df = 70, p-value = 0.6958) (Figure 2.5). The 70 overlapping taxa found in at least two parental lines are also highly detected in the G0 dataset, with 61 of these ASVs found in the G0 samples, and they are very taxonomically diverse, identifying with 32 different bacterial Genera (16 ASVs were unresolved at the Genus level or labelled “Uncultured”), 39 bacterial Families, and 26 Orders (Figure 2.5, Table S2.2). The most prevalent overlapping ASVs are also highly abundant in the combined G1-G2 dataset (Figure 2.5) and the community profiles of these 70 ASVs at the Genus level are very similar across parent lines in G2 (Figure S2.5).

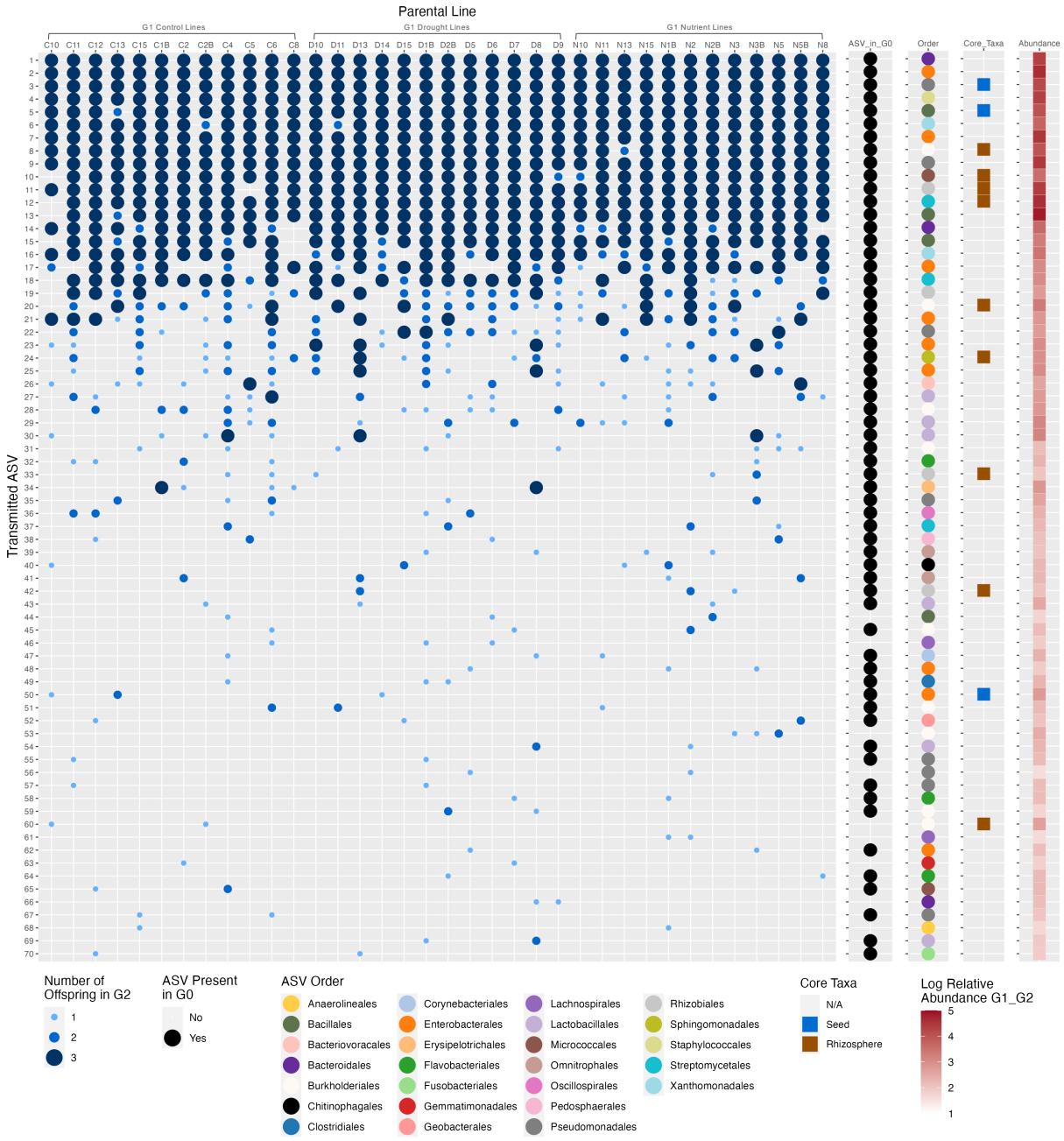

Figure 2.5. Prevalence in lines, transmission, core taxa identity and relative abundance of overlapping ASVs in G2 offspring. Of the 99 taxa that were found overlapping between G1 and G2 within parent lines, 29 ASVs that were only in one parent line were removed, and the remaining 70 ASVs are listed above, ordered from presence in the highest number of lines to lowest number of lines. Blue dots represent the number of G2 offspring containing the ASV in each line. 61 of these taxa are also found in the G0 dataset indicated by black dots. The taxonomy of each ASV identified at the Order level is indicated by colored dots in the Order column (26 Orders). Blue and brown squares in the Core_Taxa column indicate identity with seed or rhizosphere core taxa, respectively. Red boxes in the right-most column represent the log relative abundance of the ASV overall across G1 and G2. There is no significant difference in

Figure 2.5 (cont'd)
ASV transmission in G2 offspring between G1 treatments or parental lines (Pearson's Chi-squared Test. G1 Treatment: X^2 = 0.67413, df = 4, p-value = 0.9545. Line: X^2 = 63.48, df = 70, p-value = 0.6958).

While parental plant line was a strong driver of differences in the beta diversity of the entire G2 dataset, it is more pronounced in the drought-treated parental lines than the control and nutrient lines. To investigate a potential mechanism of this variation, the ASVs were queried for taxa that are only found in one parental line from each treatment. Of these taxa, there were 22 taxa unique to only one G1-G2 overlap in control lines, 26 unique taxa in nutrient lines, and 38 unique taxa in drought lines, suggesting that there are more unique taxa and more distinct communities transmitted from parent plants that experienced the drought stress treatment (Figure S2.6). Of these unique taxa within lines, there were multiple ASVs that were only found in lines from one G1 parent treatment (Figure S2.6). These unique taxa in each treatment condition were diverse at the Genus and Family level, but the taxa found only in the control lines were mostly from the phylum Firmicutes, while the nutrient and drought treatments had more unique Actinobacteriota and Proteobacteria (See Table S2.3 for a list of the taxonomy for all unique taxa within treatments). Since there were no significant differences in beta dispersion between parent treatments or lines, these results suggest that differences in beta-diversity between parental lines under the drought treatment are primarily due to differences in community composition verses variability between offspring within lines.

These results indicate that the prevalent seed microbiome members found in most parental lines in this study were consistently packaged in the seed microbiome of the G0, G1 and G2 plants, regardless of the abiotic treatment applied to the plant. While there was a stable signature of the most common and abundant overlapping seed microbiome taxa, there were

also many taxa that were only found in one or a few parental lines, and there were more unique taxa identified between the G1 drought stress lines than in the other treatment groups.

Table 2.1

Stable ASVs that were detected in all three generations. Nine ASVs are found in all 36 parental lines, two of which are core seed microbiome taxa, labeled "S". 13 additional ASVs were present in 50% or more of the parental lines. An additional core seed microbiome member was found in three parental lines. Five of these ASVs align to bean rhizosphere core OTUs at >96% identity, labeled "R". All ASVs listed are also found in Generation 0 seeds.

ASV	Presence	Core Taxa	Class	Order	Family	Genus
1	All lines	-	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Porphyromonas*
2	All lines	-	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	
3	All lines	S	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
4	All lines	-	Bacilli	Staphylococcales	Staphylococcaceae	*Staphylococcus*
5	All lines	S	Bacilli	Bacillales	Bacillaceae	*Bacillus*
6	All lines	-	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*
7	All lines	-	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Escherichia-Shigella*
8	All lines	R	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	*Massilia*
9	All lines	-	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
10	35 lines	R	Actinobacteria	Micrococcales	Micrococcaceae	*Arthrobacter*
11	35 lines	R	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Ochrobactrum*
12	35 lines	R	Actinobacteria	Streptomycetales	Streptomycetaceae	*Streptomyces*
13	35 lines	-	Bacilli	Bacillales	Bacillaceae	*Bacillus*
14	34 lines	-	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*
15	34 lines	-	Bacilli	Bacillales	Bacillaceae	*Bacillus*
16	32 lines	-	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Xanthomonas*
17	28 lines	-	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	
18	28 lines	-	Actinobacteria	Streptomycetales	Streptomycetaceae	*Streptomyces*
19	27 lines	-	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Ochrobactrum*
20	24 lines	R	Gammaproteobacteria	Burkholderiales	Comamonadaceae	
21	23 lines	-	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Escherichia-Shigella*
22	18 lines	-	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
50	3 lines	S	Gammaproteobacteria	Enterobacterales	Erwiniaceae	*Pantoea*

Previously identified core bean microbiome taxa are present in all three generations.

The 70 overlapping ASVs identified in this study were compared to the six core common bean seed microbiome ASVs identified by Simonin *et al.* 2022[50], and to the 48 core common bean rhizosphere OTUs identified by Stopnisek and Shade 2021[84] (Figure 2.5). The 22 most prevalent ASVs identified in the dataset are listed in Table 2.1 with their prevalence across lines, identity to core bean microbiome taxa, and most resolved taxonomic identification (Table 2.1).

Three core common bean seed ASVs were found in the overlapping G1-G2 dataset, specifically from the *Pseudomonas*, *Bacillus*, and *Pantoea* genera (Table 2.1, Figure S2.4)[50]. Two of these ASVs, the *Pseudomonas* and *Bacillus* core members, were identified in all parental lines the dataset, while the *Pantoea* core seed microbiome member was found in only three parental lines (Table 2.1).

To compare to the core rhizosphere OTUs, the full seed dataset was BLASTed against the core rhizosphere fasta sequences at >96% identity (the OTU identity threshold used for the core study), and there were 579 total hits to the core OTUs, primarily in the phyla Actinobacteria and Proteobacteria. Further, when the overlapping ASVs from our study were compared to the BLAST hits, there were nine taxa aligned with core rhizosphere OTUs at >96% identity (Figure 2.5, Table 2.1, Table S2.2)[84]. These taxa were classified in the families Oxalobacteraceae (genus *Massilia*), Comamonadaceae, Rhizobiaceae (genus *Ochrobactrum*), Streptomycetaceae (genus *Streptomyces*), Sphingomonadaceae (genus *Sphingomonas*), Methyloligellaceae, Devosiaceae (genus *Devosia*), and Micrococcaceae in the genus *Arthrobacter*, of which our seed ASV aligned at 98.8% identity to the most abundant core OTU

in the rhizosphere study (Table 2.1)[84]. Additionally, three unique Proteobacteria found in drought lines, including two in the order Rhizobiales, aligned at 97% identity to core rhizosphere OTUs from Stopnisek and Shade 2021[84] (Table S2.3). Along with the core seed microbiome members identified in the dataset, this provides evidence that the bean core microbiome is vertically transmitted from parent to offspring via the seed.

DISCUSSION

Our multigenerational study aimed to understand the impact of abiotic treatment on the seed microbiome in common bean over multiple generations, and the potential vertical transmission of seed microbiome members under these conditions. Since the seed microbiome can be an important factor in the establishment of the plant microbiome, it is important to understand which seed microbiome members are being passed from parent to offspring, and what advantage or disadvantage these seed members may bring to the next plant generation.

Motivated by the work of Bintarti et al., we hypothesized that the seed microbiomes of the common bean plants in our study would be significantly altered by the drought or nutrient treatment applied[44]. However, there was no statistically supported impact of the plant treatments on the seed microbiome communities in either plant generation. These results differ from what was observed by Bintarti et al. However, with the known intrinsic variability of the seed microbiome, our experiment was designed to have high replication within the treatments for increased statistical power[49]. An additional improvement to the previous experiment was the use of representative agricultural soil for plant growth with naturally occurring soil microbiota, as opposed to a greenhouse soil. It is important for greenhouse and growth chamber studies to be as realistic as possible to true field systems, and our study design

allowed for this with a highly diverse soil community[86]. This study also incorporated improved sequencing depth of the bacterial community in the seed. While our first hypothesis regarding the impact of environmental treatment was not supported, this study sheds light on the stability of the microbiome that is packaged in the seeds of common bean under varying environmental conditions, and a potential mechanism for core microbiome members to consistently associate with plant species despite varying climates and growing regions[84,87].

A significant finding of this study was the strong evidence of vertical transmission from specific parental plant lines. There were many ASVs present in the seeds of all three plant generations that were highly abundant across the dataset, including the 22 prevalent taxa identified in the parental lines. These taxa were consistently identified in parents and offspring through the generations, despite the plants being grown in the field in G0, receiving different abiotic treatments, growing in soils that were collected from the field at different times in G1 and G2, and the three G2 offspring receiving different abiotic treatments within the same parent line. It has been shown previously that common bean seed microbiomes are closely associated with the vascular system of the plant verses floral compartments, so we can be confident that these taxa were not deposited from the growth chamber system through flowers and were transported to the seed via the parent plant vascular tissues[21,29]. Additionally, the specificity of ASVs assigned at 100% sequence identity, and the thorough use of sequencing controls, allows for confidence that the taxa identified are the same across the different generations and samples[88]. Since the specific parent plant was the most significant driver of differences in the G2 seed microbiome communities, responsible for 35% of the variation in the PERMANOVA analysis, these results provide important insight to the field that parental plant

line should be considered in seed microbiome experiments, and replication within treatments and parent lines is important when designing microbiome studies. This is in line with previous studies that also reported differences in seed microbial communities based on parental plant line in *Setaria* and oak[19,43]. Our second hypothesis that characteristic taxa would be transmitted from parent to offspring under specific abiotic treatments was not fully supported. However, there is clear evidence that a stable seed microbiome was transmitted in all treatment conditions in our study.

In addition to parent plant line driving variation in the second generation, there were also many taxa found in this study that have previously been described as "core" microbiome taxa for common bean. Within the most prevalent taxa in our study, there were two core bean seed ASVs and five core bean rhizosphere OTUs identified across all three generations[50,84]. These core taxa that have been identified across multiple studies are likely important for plant health in common bean, and are thus consistently transmitted to each new plant generation. The transmitted taxa in our study were highly prevalent across parent lines and were highly abundant in the dataset, comprising 17% to 45% of the ASVs in each parent line. At such high prevalence across the dataset despite parental line treatment, these taxa, particularly the 22 most prevalent ASVs, appear to be very stably transmitted from the parent plants to offspring. These prevalent taxa include many genera that are commonly associated with plant microbiomes, such as *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Massilia*, *Stenotrophomonas*, *Ochrobactrum*, and *Xanthomonas*, which are often associated with beneficial traits such as plant growth promotion, biocontrol of pathogens, and stress resistance[34,89–94]. These stable seed microbiome taxa likely establish mutualistic or commensal associations in the microbiome

of the germinating seedling, and persist through the plant lifecycle until they are packaged within the seed for the next generation. However, an alternative mechanism that cannot be ruled out by this study, since we did not track specific bacterial cells from the seeds, could be that the parent plants are selectively recruiting these same taxa from the soil in each generation, and they then are packaged inside the seed through the vascular system[95].

The prevalent taxa in this study should be investigated further for potential use in plant microbiome engineering, as they may provide important benefits for plant health[96,97]. It has been demonstrated that applying beneficial plant microbiome members through seed treatments or soil inoculation can confer improved growth and health benefits to plants[98,99] As the need for sustainable solutions to increase agricultural productivity grows, microbiome engineering and breeding plants for improved microbiomes will continue to be an important area of research[100,101]. The stable bean seed microbiome members identified here, as well as core beneficial seed microbes identified in other plant species, are an excellent starting point for future research into plant microbiome engineering[50,102,103].

While the stable prevalent taxa in this study could be identified in parent lines from all treatment groups, there were also unique taxa identified between parent lines, and the greatest number of unique taxa were identified in the drought parental lines. These taxa were only found in one parental line and had low relative abundance in the dataset overall. This increased prevalence of rare taxa, creating more distinct communities between parent lines in the drought treatment, may be driving the increased influence of the parent on the seed microbiome communities under drought stress. It has been documented that environmental disturbance can alter microbial communities, and drought can cause significant perturbation of

plant microbiomes[10,104,105]. Additionally, more variable and diverse microbial communities are also known to be more resilient to environmental disturbances[106,107]. The parental plants that experienced a stress treatment accumulated more variable communities in their seeds, had an overall increase of rare taxa in the parent lines, and included additional taxa that were related to core bean microbiome members[50,84]. This may indicate that stressed plants contain enriched beneficial core taxa in their seeds that can persist in the offspring plant, and may be accumulating increased diversity that could be beneficial over longer time scales. While we did not observe a direct influence of plant treatment on the seed microbiome within generations, it is possible that over many more generations of stress, the increased variability and core taxa members may lead to significant shifts in microbiome communities.

In conclusion, this study provides evidence of vertical transmission of stable microbiome members in the seeds of common bean. We found that parental plant line was highly explanatory in driving the seed microbiome of the second plant generation, and this effect was heightened when the parent plant experienced a stress treatment. There were many prevalent seed microbiome members stably transmitted through three plant generations despite abiotic treatment, and these prevalent taxa included known common bean seed and rhizosphere core taxa. The prevalent taxa identified here could be an area of interest for microbiome engineering for the sustainable support of agricultural crops, and should be investigated further. Parental plant has been demonstrated to be an important factor influencing the seed microbiome and should be included as an experimental variable when investigating the plant microbiome. Overall, this study contributes important knowledge to the field of seed microbiome research

to inform future studies, and contributes to a significant knowledge gap in the transmission of endophytic seed communities under stress conditions.

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APPENDIX B:
CHAPTER 3 SUPPLEMENTARY MATERIALS

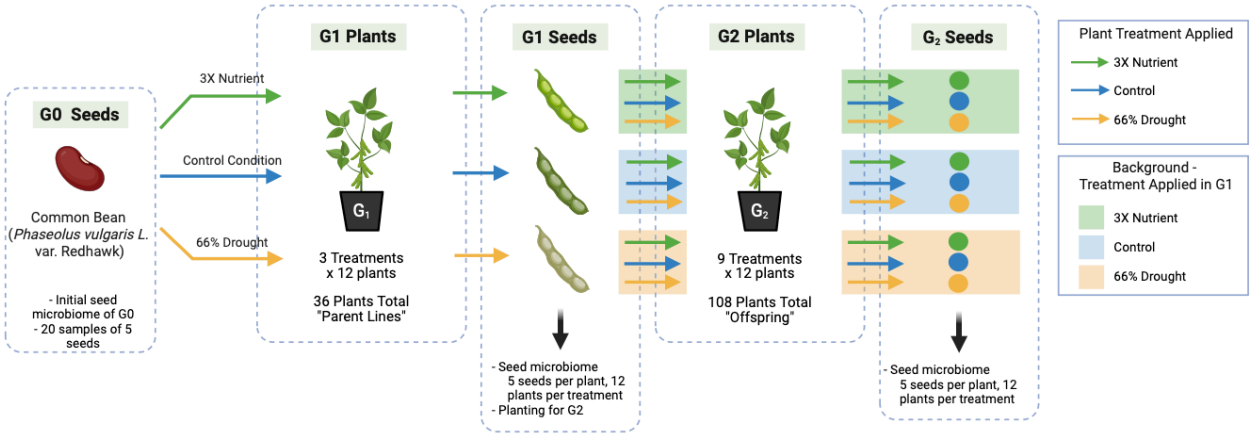

Figure S2.1. Experimental Design. Seed microbiome samples were taken from the G0 seed pool and G1 and G2 plants. Treatments were applied in G2 in a full factorial design, where one offspring from each G1 parent line was treated with each of the three treatments in G2.

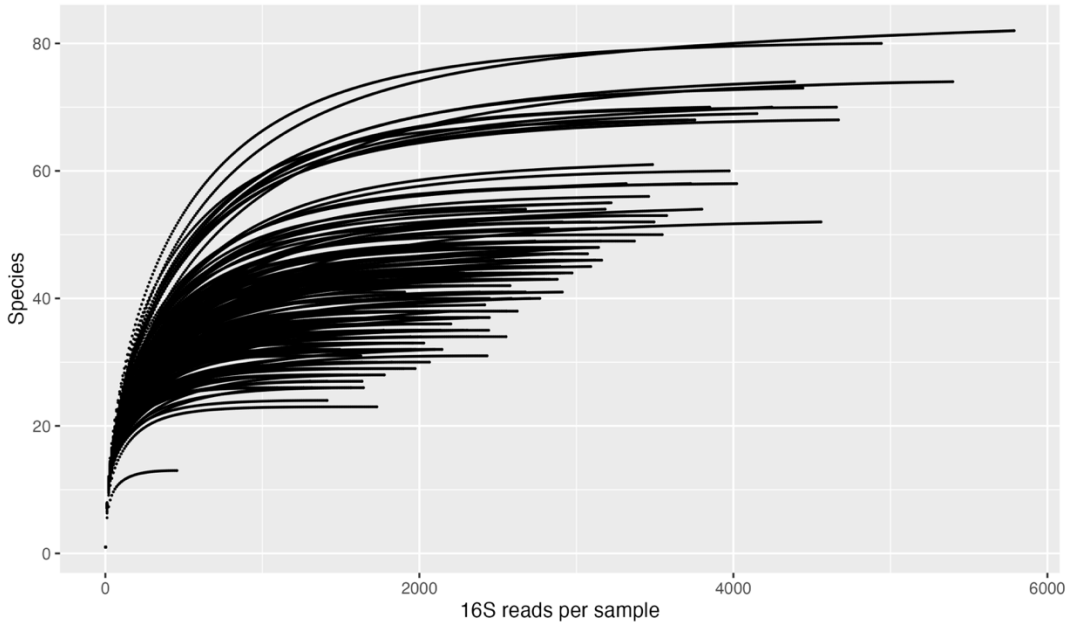

Figure S2.2. Rarefaction curves of quality-filtered microbiome profiles (host reads removed, see methods). Each line represents one seed microbiome sample (pool of 5 seeds from the same parent plant). The DNA read range is 456-5788.

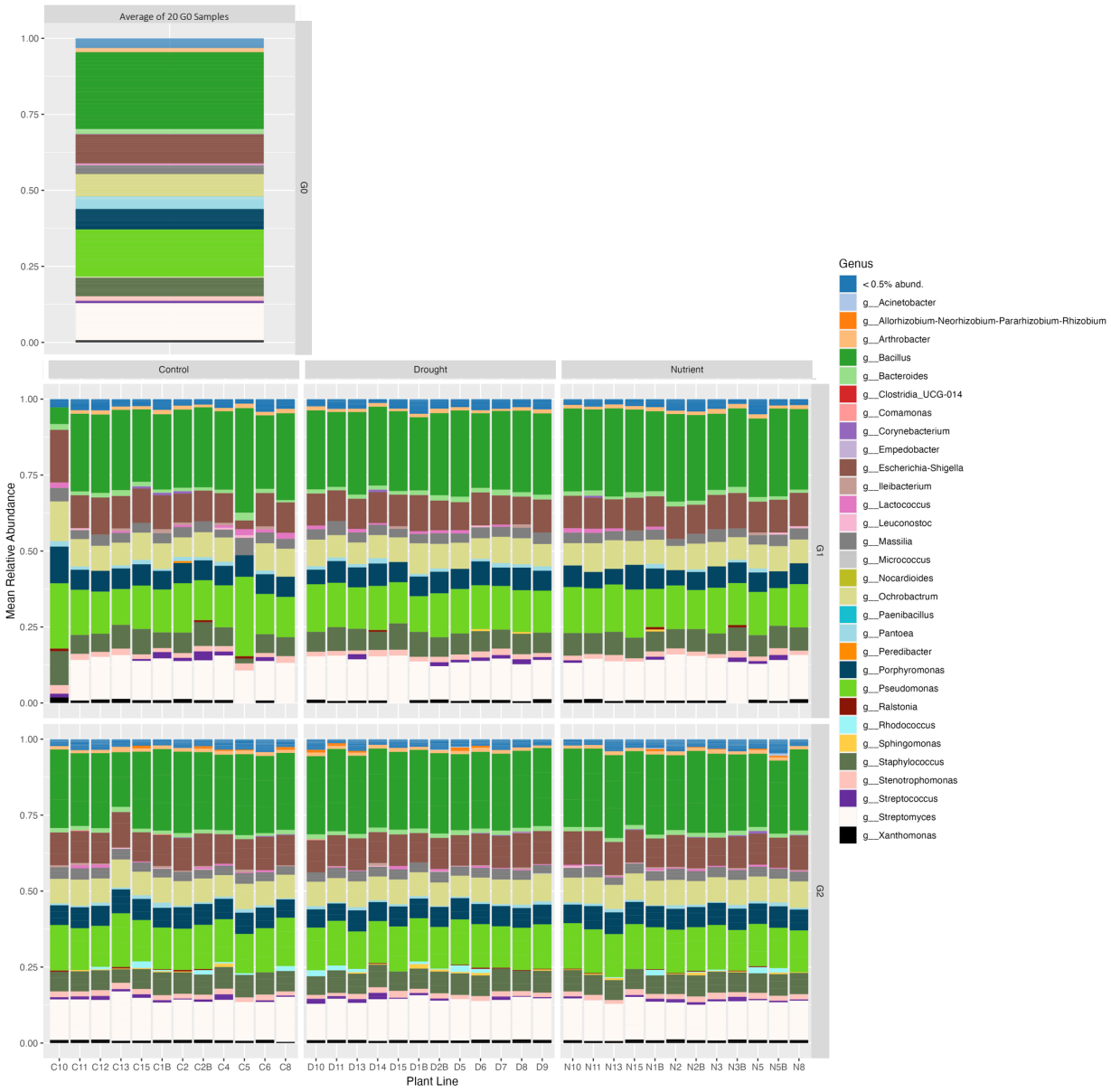

Figure S2.3. Mean relative abundance of full dataset ASVs identified at the genus level across all three generations. The bar in the top row is the average of the 20 seed samples from G0. Bars in the middle row represent G1 parent samples. Bars in the bottom row represent the average of the 3 G2 offspring samples in each parent line. The "< 0.5% abund." category is comprised of 263 genera that are less than 0.5% abundant in the dataset.

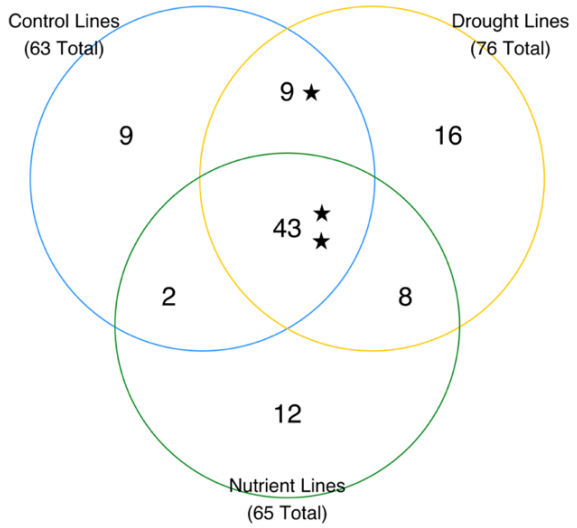

Figure S2.4. Number of ASVs found overlapping between G1 and G2 within parent lines and shared between parent treatment groups. Stars indicate the presence of core seed microbiome taxa. There are 99 total taxa represented, of which 85 can also be found in G0.

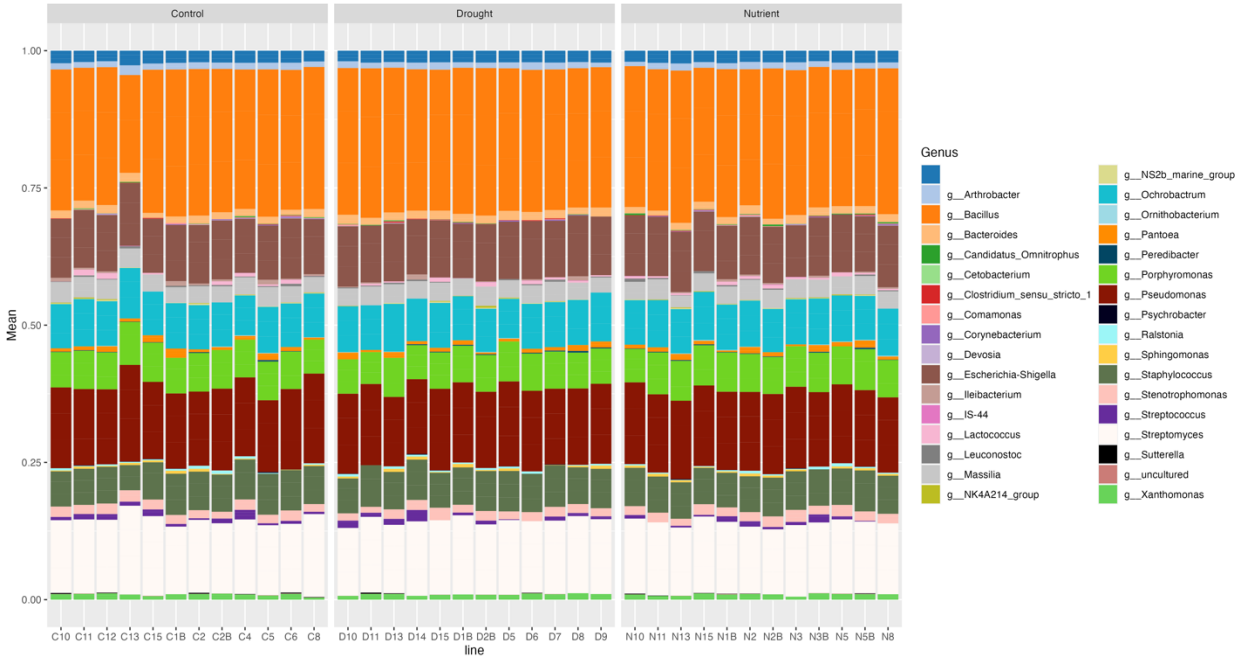

Figure S2.5. Mean relative abundance of 70 prevalent overlapping ASVs across parent lines in G2 identified at the genus level. All other ASVs have been removed from the dataset. The unlabeled legend color represents ASVs that are unresolved at the genus level.

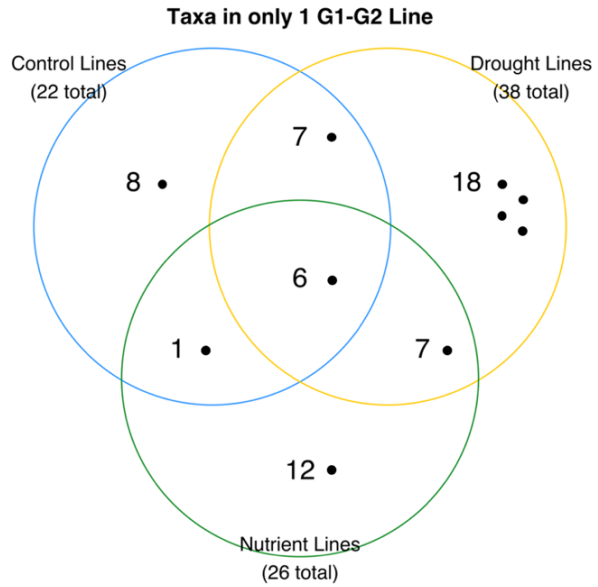

Figure S2.6. Number of ASVs found overlapping in G1 and G2 in only 1 parental line in each treatment group. There are 59 unique taxa represented, 49 of which can be found in G0. Dots represent taxa that are not found in the G0 dataset.

Table S2.1 Supplemental File: Supplement metadata table with plant treatment data and extraction batches

Table S2.2 Supplemental File: List of 70 overlapping taxa with taxonomy

Table S2.3 Supplemental File: Supplemental table of unique taxa

CHAPTER 4:

Elucidating the recruitment timing and development of the root microbiome over the common bean lifecycle

ABSTRACT

Plant microbiomes play an important role in the health of the plant, particularly in the rhizosphere and rhizoplane communities. Feedbacks between the growing plant and microbial cells in the soil contribute to the assembly of microbial communities over the life of the plant, but it is difficult to separate the influence of time from plant development in these environments. We conducted an innovative experiment utilizing zebularine treatment, a DNA methylation inhibitor, to induce a growth delay in common bean plants. This allowed us to investigate the influence of plant growth stage and time across the common bean life cycle. We identified closely aligned bacterial communities in the rhizoplane of common bean treated with and without zebularine based on plant growth stage, despite the plants growing at different rates. Plant growth stage had a stronger influence on the bacterial communities than time, while a large proportion of the community variation was attributed to neutral processes. Indicator taxa associated with plant growth stages were identified and found to be under a selective pressure in the common bean rhizoplane. Selected ASVs included known beneficial plant microbiome taxa such as genera *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Pseudolabrys, Duganella, Paenibacillus, Bacillus, Thermoactinomyces* and *Gaiella*. These taxa that were under selection in the plant microbiome environment may provide valuable insight into key microbiome members that could be beneficial to plant health.

INTRODUCTION

The root and rhizosphere microbiome plays a critical role in plant health and supports the plant by providing important benefits like growth promotion and resilience to biotic and abiotic stress[1]. Particularly in the closely associated soil around the plant root, bacterial and fungal communities can assist with water assimilation and nutrient uptake, can out-compete pathogenic species, and provide plant growth promoting chemical feedbacks[2–5]. In the roots of leguminous plants such as common bean (*Phaseolus vulgaris L.*), roots also form symbiotic relationships with rhizobia species within their root nodules, which perform symbiotic nitrogen fixation and provide usable nitrogen to the plant[6–8]. Plants also produce root exudates such as phytohormones in the soil that impact microbial communities and can enrich specific bacterial and fungal species that are beneficial for the plant[9–11].

The microbial communities in the root and rhizosphere are dynamic and diverse. Many factors have been investigated that can impact these communities including plant genotype, abiotic stress such as drought, soil properties, temperature, and the presence of plant pathogens[12–16]. Additionally, it has been documented that the root microbiome changes over the life cycle of the plant in various plant species such as maize, canola, and the medicinal plant *Panax notoginseng*[17–19]. Alterations in the rhizosphere microbiome over time can further support the health of the plant, such as through increased resistance to pathogens[3]. The microbial communities in the plant root system typically become more taxonomically and functionally diverse as the plant grows, and shift in community composition can coincide with shifts in plant life stages[3,20,21].

While there are various factors that can influence root and rhizosphere communities, a key factor in plant microbiome assembly is the influence of the plant via interactions such as the release of phytohormones and plant-microbe feedbacks[22,23]. Root exudates can influence the plant growth-promoting bacteria in the rhizosphere, contribute to the establishment of symbiotic associations, and drive community assembly of the microbiome with plant growth[10,18,24–27]. However, there are also other factors that can influence microbial communities in soil, such as soil and environmental conditions and the natural succession of microbial species over time [28–31]. Because plant growth and time are closely linked, it is difficult to study the influence of these two variables separately in the plant microbiome. Studies have investigated the temporal variation of plant microbiomes over the growing season or plant life cycle, but these studies are unable to determine the proportion of microbial change attributed to community progression over time versus the influence of plant development[22,32].

This study aimed to tease apart these two variables, plant growth and time, in order to determine the proportion of the community succession that could be attributed to these variables, and which variable was the primary driver of community assembly in common bean. We used a chemical treatment, zebularine (zeb), to induce a plant growth delay in common bean plants, which allowed us to study the microbial communities of the plant root system over two sampling schemes, plant growth stage and time since planting. Zebularine is a DNA methylation inhibitor that is often used to study epigenetic changes in plants[33]. However, an interesting result of zebularine treatment on Common Bean seeds is a significant growth delay once planted. When common bean seeds are treated with zebularine, plant growth progresses at a rate 2-3 weeks behind plants that are not treated. We grew groups of plants with and

without zebularine treatment and sampled the bacterial communities across two different sampling schemes, allowing us to separate plant growth from time.

We hypothesized that the zebularine plants sampled under the growth stage and time series sampling schemes would have distinct microbial communities, and these communities would either resemble the untreated plants at the growth stages, or the untreated plants at days since planting. This would allow us to determine which variable was driving the development of the plant root communities. We sampled both the rhizosphere (the soil around the root system) and the rhizoplane (the community closely associated with the surface of plant roots) to understand which compartment of the root community is most affected by the growth stage and time variables. Additionally, the bacterial community in the rhizoplane was assessed with the Sloan neutral model to determine the influence of selective and neutral processes in the common bean rhizoplane, and indicator taxa associated with bean growth stages were investigated. This study provides important insight into how the root community is influenced by the plant over time and the role of assembly processes in the rhizoplane.

METHODS

Surface sterilization and Zebularine seed treatment

Phaseolus vulgaris L. var. "Red Hawk"[34] seeds were surface-sterilized in a solution of 10% bleach and 0.1% Tween20, then treated with a solution of 5 mM zebularine (zeb) dissolved in Milli-Q purified water, or a control treatment with Milli-Q water only. For surface-sterilization, seeds were placed in a sterile glass beaker and covered with approximately 3 cm of bleach solution. Seeds were soaked for 10 minutes with agitation at 5 minutes, then the bleach solution was drained, seeds were briefly rinsed with sterile DI H_2O five times, and then drained

of excess water. After surface-sterilization, seeds were divided into petri dishes lined with sterile filter paper. Approximately 20-25 seeds were placed in each petri dish, allowing each seed to sit directly on the filter paper. The filter paper in each petri dish was moistened with 1 mL sterile DI H_2O, then 2 mL of 5 mM zebularine solution or Milli-Q water was dripped over the seeds in the zeb treatment or control treatment dishes, respectively. The petri dishes were stored in the dark at room temperature for three days, with an additional 2 mL zebularine or control water added to the seeds each day. The seeds were then planted on the fourth day.

Plant growth, harvest, and phenotypic measurements

Before planting, agricultural field soil was collected from the Michigan State University Agronomy Farm from a field that had previously grown common bean (42°42'57.4"N, 84°27'58.9"W, East Lansing, MI, USA). The soil was a sandy loam with an average pH of 7.2. Soil was passed through a 4 mm sieve to remove rocks and plant debris and stored in sealed buckets at 4°C prior to use. Immediately before use the soil was mixed with autoclaved coarse vermiculite at a 50% v/v ratio. Seeds were then planted in the field soil and vermiculite mixture in 2x2 inch seedling pots in a high-light BioChambers FLEX™ LED growth chamber with a 16-hour day/8-hour night cycle at 26°C and 22°C, respectively, and 50% relative humidity. Seeds were lightly covered with soil and pots were bottom watered by filling a tray beneath the pots with water and allowing the soil to absorb water until thoroughly moist. Pots were watered with half-strength Hoagland's solution once a week and DI H_2O every two or three days as needed to maintain soil moisture[35]. As seedlings grew, they were transferred to larger pots, either 4x4 inch pots for plants that would be harvested at earlier time points, and 1-gallon pots for plants that would be harvested at later time points.

Five plants from both the control and zebularine treatments were randomly selected for destructive sampling at each sample point. Plants harvested based on growth stage were harvested at the following stages: V1 - first trifoliate leaves expanded, V2 - second trifoliate, V3 - third trifoliate, V4 - fourth trifoliate, R1 - first open flowers, R4 - approximately half of the seed pods fully developed, R7 - seed pods dry and plant senescing[36]. Plants harvested based on the time series were harvested on the following days after planting: 3, 7, 14, 21, 35, 49, 63. The time series harvest days roughly aligned with the control treated growth stage sample points. See Figure S3.1 for experimental design schematic. Before harvest, pot trays were emptied of water to allow the soil to dry for 2-3 days. On harvest day, plant height was measured with a ruler from the surface of the soil to the tallest part of the plant, holding the plant upright to its tallest point if it had fallen over. For day 3, a sterilized scoopula was used to scoop out the soil closest to the roots of the seedling to be collected as rhizosphere. At all other sampling points, the plant and soil were turned out of the pot into a clean tray. The root system was gently removed from the soil, placed in a sterile Whirl-pak® bag, and shaken to collect the rhizosphere soil. Then the roots were placed on a white background next to a centimeter ruler to be photographed for root analysis. Next, the above-ground biomass was cut from the root system and placed in a paper envelope, and the root system was placed in another sterile Whirl-pak® bag. The root and rhizosphere samples were flash-frozen in liquid nitrogen and then stored at -80°C until DNA extractions were performed. The above-ground biomass was weighed in its envelope immediately after harvest for fresh weight, then placed in a 50°C drying oven for 2-3 days and weighed again for dry weight.

Root photos were analyzed with ImageJ software. For each photo, the scale of the image was set in millimeters using the ruler in the image, and the freehand line tool was used to trace and measure the five longest roots starting from where the stem met the soil. The average root length was calculated for the plant based on the five length measurements. For zebularine plants at early time series sampling points, only the main taproot was measured due to lack of lateral roots. Traced paths of roots were estimated for mature plants with dense root systems. Visible root nodules were counted in each photo with the ImageJ multi-point tool. Nodule count is an estimate based on what was visible in the root photo and does not necessarily account for nodules in the entire root system, as some nodules may not have been visible.

Bacterial DNA extraction for microbiome analysis

Rhizosphere and root samples were processed in randomized batches, see supplemental metadata table for extraction batch information. Root samples were thawed at room temperature, and a 4-5 cm section of the "active" outer zone of the root system (or the entire root system for very small plants) was cut with a sterilized razor blade and placed in a sterile 50 mL conical tube with enough sterile DI H_2O to cover the roots. The roots were gently swirled to remove loosely adhered soil, then transferred to a sterile 15 mL conical tube with enough sterile Phosphate Buffered Saline (PBS) + 0.05% Tween20 to cover the roots. Very small roots were not rinsed with water and were placed directly in the 15 mL conical tube due to low surface area with a small amount of adhered soil. Roots were then vortexed at maximum speed in the PBS solution for 1-2 minutes until all rhizoplane soil was removed from the surface of the roots. Root material was then removed, and the tubes were centrifuged at 4600 x g, 4°C, for 15

minutes to pellet the rhizoplane material. Following centrifugation, the PBS supernatant was removed and pellets were stored at -20°C until DNA extraction.

DNA extraction was performed with the DNeasy PowerSoil Pro DNA Kit (Qiagen, Germantown, MD, USA). Rhizosphere soil was thawed at room temperature and weighed into the bead beating tubes for the first step of the DNA extraction protocol. Rhizoplane pellets were thawed on ice and then weighed into the bead beating tubes. DNA extraction was performed following manufacturer's instruction with the following modifications: In step one, 30-50 µg of sample was used in each tube, then 700 µL solution CD1 was used with 100 µL ATL buffer (Qiagen, Germantown, MD, USA). The bead beating step was performed for 15 minutes on a vortex genie 24-tube adapter at maximum speed. Lastly, 60 µL of final elution buffer C6 was used, and tubes were incubated at room temperature for 10 minutes before centrifugation. Each extraction batch included a negative control of an empty PowerSoil Pro tube at the start of the extraction protocol. For the rhizoplane negative controls, solution CD1 was swirled in a sterile 15 mL conical tube before being added to the PowerSoil Pro tube. A positive control of a custom-made mock bacterial community suspended in solution CD1 was used in one extraction batch per compartment[37]. Controls were processed with the extraction batch and then sequenced with the true samples for use in downstream DNA sequence decontamination.

Sequencing and sequence processing

DNA sequencing was performed by the Michigan State University Research Technology Support Facility Genomics Core in East Lansing, Michigan, USA, following their standard protocol. The V4 region of the 16S rRNA gene was amplified from the rhizosphere and

rhizoplane extractions using dual indexed, Illumina compatible primers following the protocol described in Kozich, JJ, et al. (2013)[38]. PCR products were batch normalized using an Invitrogen SequalPrep DNA Normalization plate and product recovered from the plates pooled. The pool was concentrated and cleaned up using a QIAquick Spin column and AMPure XP magnetic beads. The pool was QC'd and quantified using a combination of Qubit dsDNA HS, Agilent 4200 TapeStation HS DNA1000 and Invitrogen Collibri Illumina Library Quantification qPCR assays.This pool was loaded onto one (1) MiSeq v2 Standard flow cell and sequencing was carried out in a 2x250bp paired end format using a MiSeq v2 500 cycle reagent cartridge. Custom sequencing and index primers complementary to the 515f/806r oligomers were added to appropriate wells of the reagent cartridge. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.20.0. Sequencing was repeatedly performed in three MiSeq runs to maximize the total number of DNA reads returned for the dataset.

After sequencing, fastq files were processed in QIIME2 after primer removal by the sequencing center (QIIME2 version: 2022.8.0)[39]. Sample fastqs were imported to QIIME2 format, and samples were denoised, truncated and merged using Dada2 and amplicon sequence variants (ASVs) were defined at 100% sequence identity[40]. Customized truncation lengths for each MiSeq run were determined using FIGARO[41]. Run 1 had a forward truncation length of 99 and reverse length of 176, run 2 had forward length 124 and reverse length 151, and run 3 had forward length 125 and reverse length 150. Once representative sequences and ASV tables were created for each run, the runs were merged into one dataset and 16S

taxonomy was assigned with the Silva database release 138[42]. Taxonomy and ASV tables were then exported for further analysis in R.

Sequence Decontamination

Data analyses were performed in R version 4.3.1 and R Studio version 2023.06.1+524[43]. There were 20.1 million merged DNA reads in the dataset prior to host removal and decontamination. ASV, taxonomy, and metadata tables were imported into the phyloseq package and host reads classified as chloroplast and mitochondria were removed using the subset_taxa() command in the phyloseq package version 1.44.0[44]. Nine percent of the total DNA reads were removed as host reads, leaving 18.3 million total bacterial DNA reads. Datasets were decontaminated with the decontam package version 1.20.0 at the 0.55 threshold utilizing the specific negative controls for each extraction group, and positive extractions with the entire dataset[45]. After decontamination, there were 16.3 million total DNA reads with a range of 3,635- 151,332 reads per sample in the full dataset. Rarefaction curves were created using the rarecurve() command in the vegan package version 2.6-4[46]. Samples were rarefied to the tenth lowest number of reads in the dataset at 22,761 using the rarefy_even_depth() command in phyloseq. Rarefaction removed nine samples that did not meet the minimum number of reads.

Analysis

Plant phenotypic traits were analyzed with the base R stats command t.test() and figures were created with the ggplot2 package version 3.4.2[47]. Alpha diversity species richness was assessed using estimate_richness() in phyloseq, and figures were created using the plot_richness() command from phyloseq with ggplot2. Bray-Curtis distances were calculated with vegdist() from the vegan package and used for all analyses of beta diversity, and

PERMANOVA statistical tests were performed with adonis2() from vegan. Post-hoc analysis on the PERMANOVA results was performed with pairwise.adonis2() from pairwiseAdonis version 0.4.1[48]. Beta diversity ordinations were created with ordinate() from phyloseq with ggplot2. Beta dispersion was assessed with the betadisper() and permutest() commands from the vegan package. Datasets were divided into groups where applicable using the ps_filter() command in the microViz package version 0.10.10[49]. Procrustes concordance analysis was performed using the vegdist(), monoMDS(), procrustes() and protest() commands in vegan. Heatmap was created using the ComplexHeatmap package version 2.16.0[50,51]. Indicator taxa were identified with the multipatt() command from the indicspecies package version 1.7.14, and the analysis was performed on all ASVs that had greater than 100 reads in the dataset[52]. The Sloan neutral model fit was performed on all ASVs with at least 5 reads in the dataset with the fit_sncm() command from the reltools package version 0.1.0[53].

Data Availability

Data analysis code and plant phenotypic measurement data can be found at (https://github.com/ShadeLab/Rhizosphere_assembly_Common_Bean). Raw DNA sequences can be found on the NCBI Sequence Read Archive under BioProject accession number PRJNA1066866. Root photographs can be accessed on figshare.com under the following DOIs: doi.org/10.6084/m9.figshare.25054997, doi.org/10.6084/m9.figshare.25055087, doi.org/10.6084/m9.figshare.25055774, doi.org/10.6084/m9.figshare.25057067.

RESULTS

Quantifying the Zebularine Growth Delay

When the common bean plants were grown with and without zebularine treatment, the zeb plants harvested based on the growth stage sampling points were at the same stage as control plants, but were harvested 7-14 days after the control plants at the same growth stage (Figure S3.2, S3.3). Alternatively, at the time series sampling points, the control treated plants were at comparable growth stages to the control plants harvested based on growth stage, while zebularine (zeb) treated plants had delayed growth compared to the control (Figure S3.2, S3.3). Above and below-ground phenotypic traits, including the plant height, fresh and dry shoot biomass, root length, and approximate nodule count, were measured at each sampling point. While the zeb treated plants did not reach the same mature height as the control plants, the above-ground fresh and dry biomass were similar between control and zeb in the growth stage series, with no significant difference between control and zeb dry biomass at plant maturity (Figure 3.1, Welch Two Sample t-test: Control Vs Zeb R4 t=-0.94992, df=7.8587, p-value=0.3704; Control vs Zeb R7, t=1.2701, df=5.5536, p-value=0.2547). Alternatively, above-ground biomass was delayed in zeb plants in the time series and zeb plants had not yet reached maturity at the final sampling point on day 63 (Figure 3.1C, Welch Two Sample t-test: Control Vs Zeb Day 63, t=7.6828, df=6.8434, p-value=0.00013***).

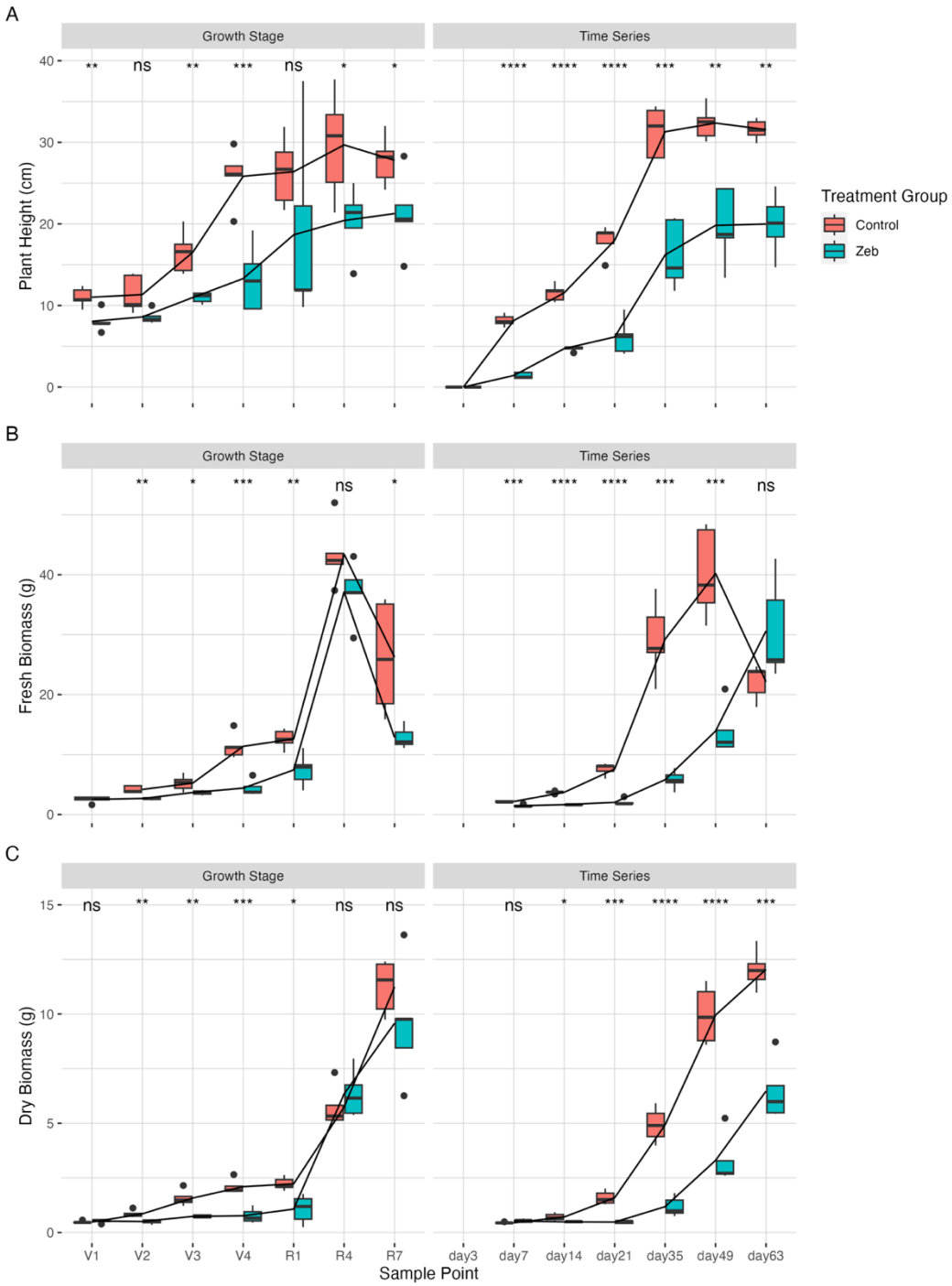

Figure 3.1. Above-ground plant growth measurements by treatment group and sampling series, Growth Stage (left) or Time Series (right). **A.** plant height (cm). **B.** above-ground fresh biomass (g). **C.** Above-ground dry biomass (g). Plants had not emerged from the soil for above-ground biomass collection at the day 3 time point. Asterisks indicate significance between Control and Zeb treatment groups at labeled sampling points. Boxplots n=5, black line indicates mean of each sample set. Welch Two Sample t-test, ns= not significant, p-values: *<0.05, **<0.01, ***<0.001, ****<0.0001.

In below-ground phenotypic measurements, similar results to plant height were observed for plant root length, where the zeb root length was shorter than control overall (Figure 3.2A, Welch Two Sample t-test: Control Vs Zeb R4, t=2.9755, df=7.6344, p-value=0.0186*; Control Vs Zeb R7, t=2.5611, df=6.1922, p-value=0.0416*; Control Vs Zeb Day 63, t=4.6969, df=7.8593, p-value=0.00162**). However, the growth delay of the zeb plants compared to control was still pronounced in the time series root length measurements (Figure 3.2A). Additionally, even though nodule count was estimated from visible nodules in root photos, counts followed a similar trajectory between control and zeb over the growth stages with no significant difference in mature plants, while nodule development was delayed in the zeb time series plants (Figure 3.2B, Welch Two Sample t-test: Control Vs Zeb R4, t=-0.42188, df=7.7992, p-value=0.6845; Control Vs Zeb R7, t=-1.217, df=7.4621, p-value=0.2607). These results confirm and quantify the observed growth delay of roughly 2 weeks when common bean plants are treated with zebularine prior to planting, which allowed us to study the root microbiome over two different developmental time series.

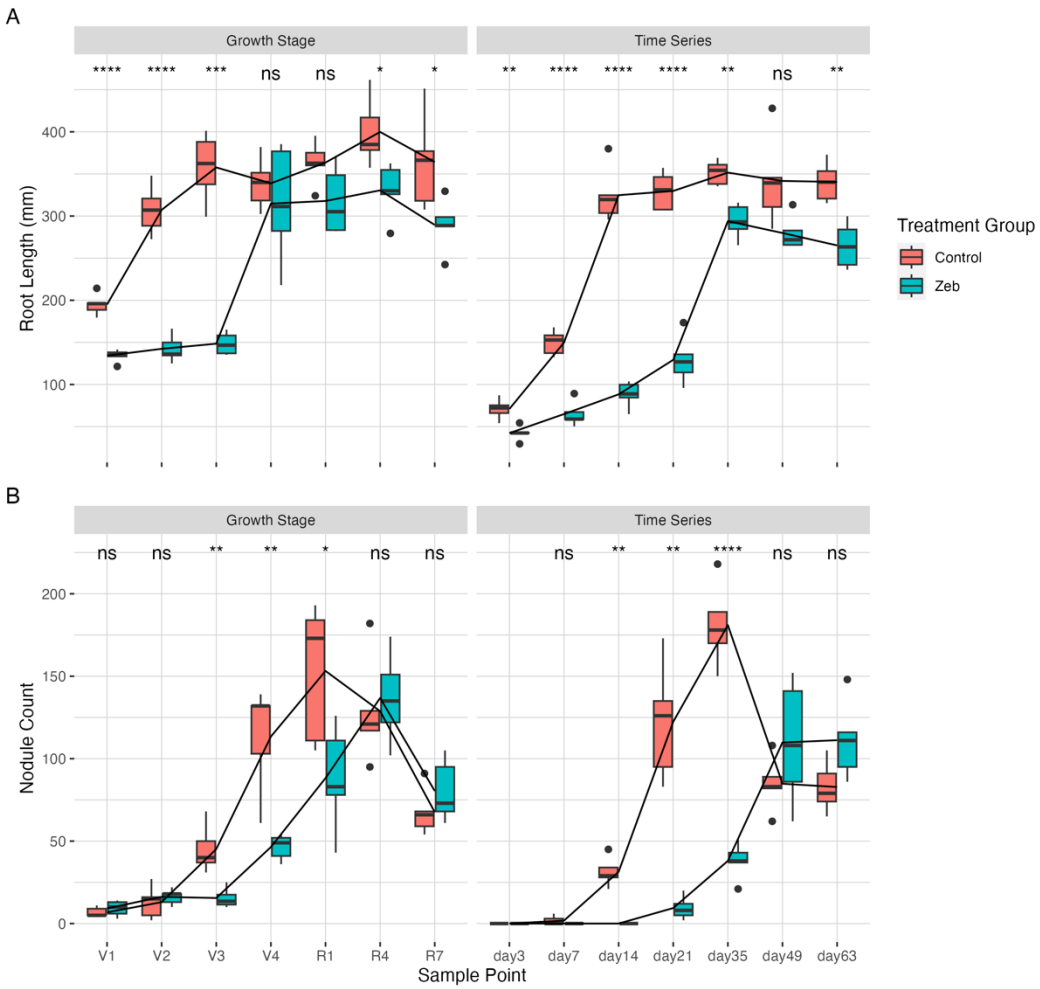

Figure 3.2. Below-ground plant development measurements by treatment group and sampling series, Growth Stage (left) or Time Series (right). **A.** average root length (mm). **B.** number of visible nodules present in root photo. Asterisks indicate significance between Control and Zeb treatment groups at labeled time points. Boxplots n=5, black line indicates mean of each sample set. Welch Two Sample t-test, ns= not significant, p-values: *<0.05, **<0.01, ***<0.001, ****<0.0001.

Rhizoplane bacterial communities are modulated by plant growth stage

Microbial communities in the rhizosphere and rhizoplane were analyzed at each sampling point with 16S V4 rRNA gene amplicon sequencing. Samples were rarefied to 22,761 DNA reads and then grouped based on plant compartment for further analysis (Figure S3.4). In the rhizosphere, ASV richness ranged from 1,000 to 2,000 ASVs per sample, and there were no significant differences between control and zeb in either sampling series (Figure 3.3). ASV

richness in the rhizoplane samples ranged from 500 to 1,500 ASVs per sample, and there was a pattern in richness over the plant life cycle. In the growth stage samples in both control and zeb treated plants, ASV richness was highest at the late vegetative and early reproductive stages (V4 and R1) and lowest when the plants reached senescence (Figure 3.4A). The control plants sampled in the time series followed a similar pattern, but the zeb treated plants in the time series had lower richness until day 35, and higher richness at later time points (Figure 3.4B). There were significant differences between control and zeb rhizoplane richness at the R4 growth stage, and the day 14 and day 35 time points (Welch Two Sample t-test, R4: t=2.6229 df=7.9952 p=0.03053*; Day14: t=2.8744 df=5.8271 p=0.02921*; Day35: t=-3.101 df=4.8484 p=0.02795*) (Figure 3.4).

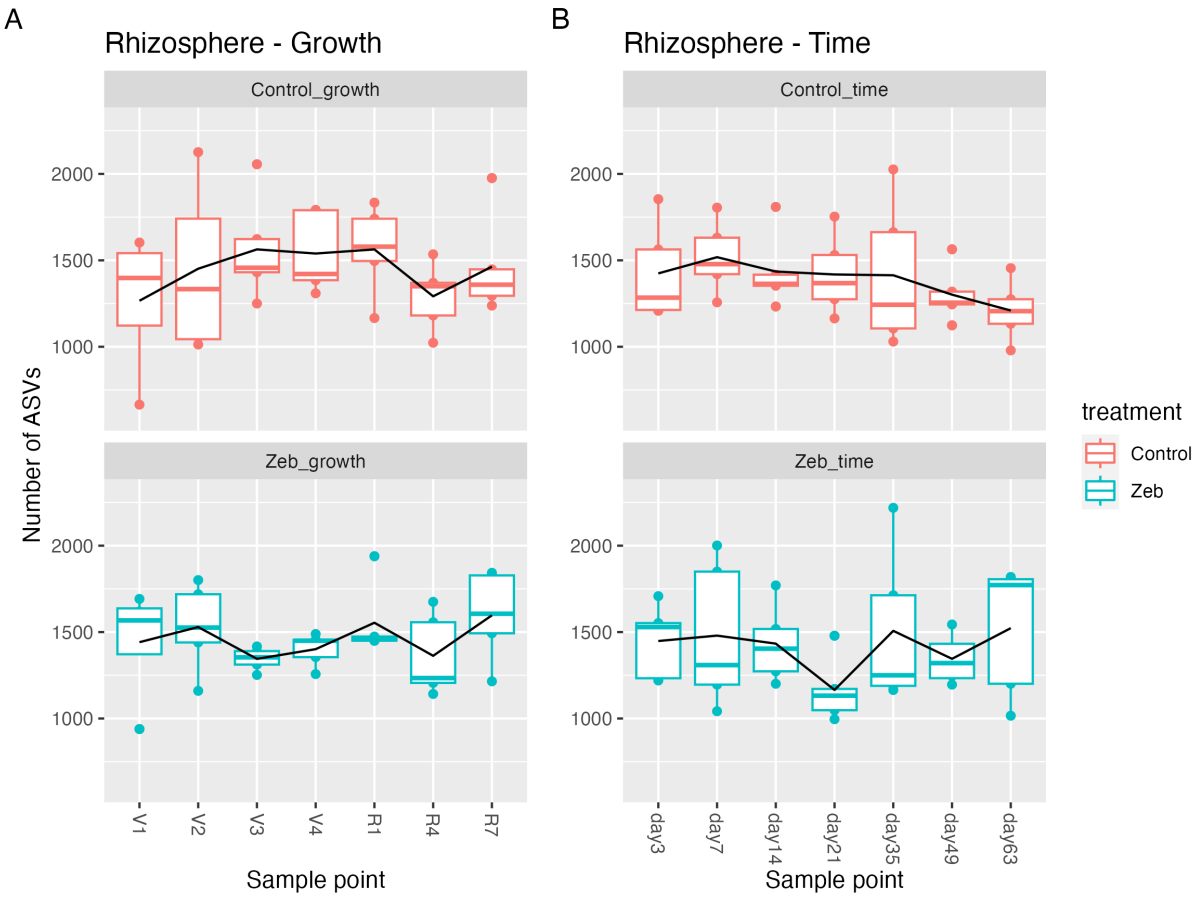

Figure 3.3. Alpha diversity in rhizosphere samples. There are no significant differences in ASV richness between control and zeb in either the **A.** growth stage series or **B.** time series. Boxplots n=5, black line indicates the mean of each sample group.

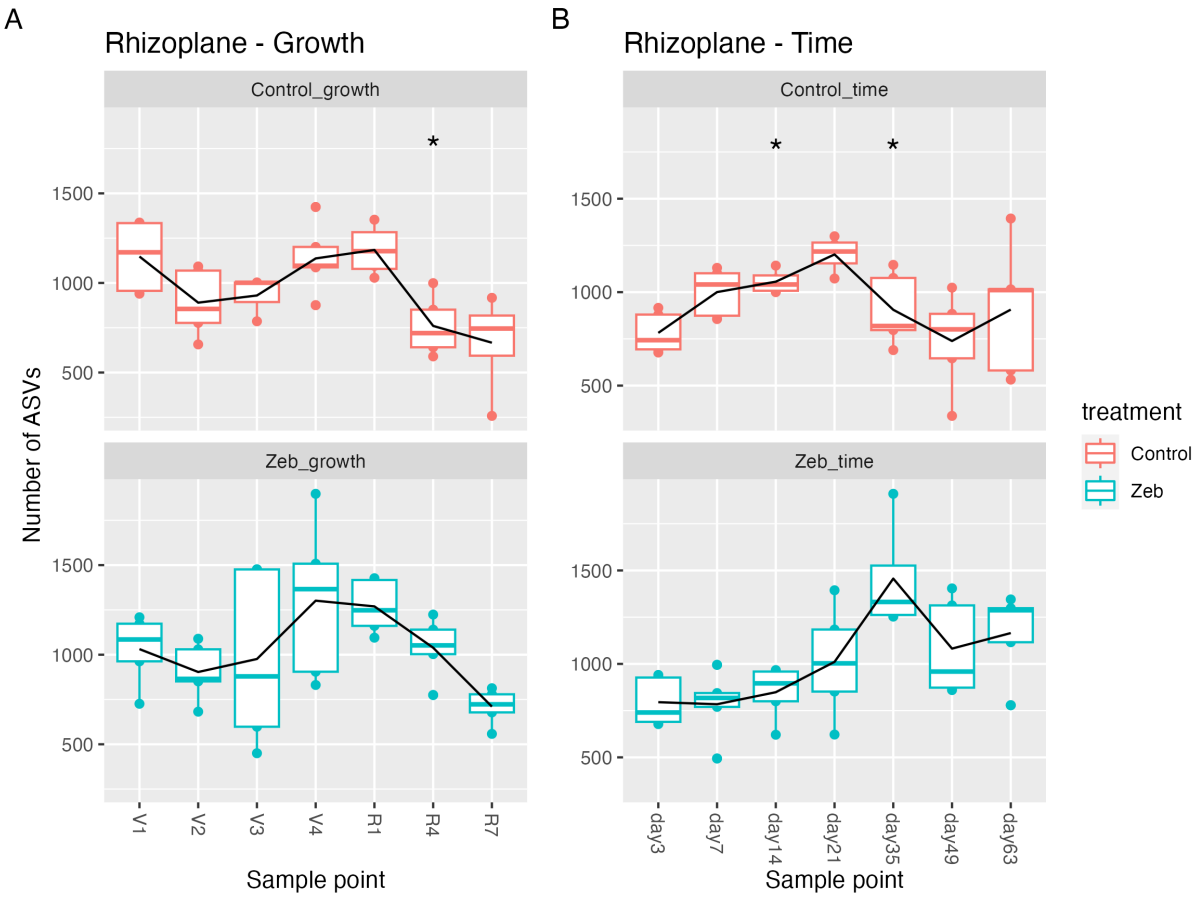

Figure 3.4. Alpha diversity in the rhizoplane. Asterisks indicate a sampling point where there is a significant difference in ASV richness between control and zeb samples within the **A.** growth stage series or **B.** time series. Boxplots n=5, black line indicates the mean of each sample group. Asterisks indicate significance between Control and Zeb treatment groups at labeled time points (Welch Two Sample t-test, R4: t=2.6229 df=7.9952 p=0.03053; Day14: t=2.8744 df=5.8271 p=0.02921; Day35: t=-3.101 df=4.8484 p=0.02795). Both growth stage groups and the control-time plants have a similar pattern of alpha diversity over the sampling points, with their highest alpha diversity at the late vegetative and early reproductive stages. The zeb-time plants do not match this pattern.

Bray-Curtis distance was used to compare the composition of the bacterial communities across sampling points. When the two control treated datasets were compared (growth stage and time), there were no significant differences based on the sampling series. However, when zeb treated growth and time datasets were compared, there were significant differences between the sampling series in both the rhizosphere and rhizoplane, further confirming that

the sampling series are not aligned in zeb treated plants, and there are differences in the microbial communities as a result (PERMANOVA, Rhizosphere Zeb Growth vs Zeb Time: r^2=0.01917, F=1.2897, p=0.038*; Rhizoplane Zeb Growth vs Zeb Time: r^2=0.03051 F=2.1087 p=0.0188*) (Data not shown, statistical results can be found in Table S3.1).

To determine which control sampling series the zeb microbial communities align with more closely, the Bray-Curtis distance was compared between the control and zeb treatment in the two sampling series. In all comparisons there were significant differences in the microbial communities based on which treatment the plants received, suggesting some influence of the zebularine treatment on the resulting root communities regardless of sampling time. However, these differences based on treatment accounted for a small amount of the variation in each dataset, and samples in the growth stage series were clustered more closely at each growth stage between control and zeb verses the samples in the time series (PERMANOVA Control vs. Zeb treatment. Rhizosphere - growth: r2=0.03035 F=2.2850 p=0.0001*, Rhizosphere - time: r2=0.02404 F=1.7894 p=0.0004*, Rhizoplane - growth: r2=0.03567 F=2.9122 p=0.0015*, Rhizoplane - time: r2=0.03382 F=3.2968 p=0.0011*) (Figure 3.5, Table S3.1). When communities were compared between control and zeb at the specific sampling points, both rhizosphere sampling series were similar at the earliest sampling points, but became significantly different from each other after the second sampling point regardless of series (Table S3.1). In the rhizoplane, the communities sampled based on the time series were significantly different between control and zeb at all time points, while the growth stage sampling points were not significantly different at the V3, V4 and R7 stages (Table S3.1). These results suggest that there is less direct influence of the plant on the rhizosphere communities in this study, while the

rhizoplane is significantly influenced by the plant growth stages, and the communities in the zebularine treated plants are more closely aligned to the control plants by growth stage verses time. When the variation in the rhizoplane dataset was investigated, 20% of the overall variance was explained by plant growth stage, 10% was explained by time (days since planting), and 2% of the variance was explained by the control or zeb treatment that the plant received (Table 3.1). There was also an interaction observed between the influence of Treatment and Growth Stage that accounted for 6% of the variation in the rhizoplane samples (Table 3.1). Since the greater proportion of variation is explained by the growth stage, this supports the observations that the rhizoplane bacterial communities are modulated by plant growth. Additionally, when the beta-dispersion of the Bray-Curtis distances were compared, there were no significant differences in distance to spatial median between the zeb and control samples in any sampling series, or between any sampling points, suggesting that the variance of the bacterial communities between samples was not affected by zeb treatment or sampling point (Figure S3.5).

Table 3.1

PERMANOVA of variance in the rhizoplane dataset. All samples were assigned a growth stage and age time point (days since planting) based on harvest date. The greatest variation in the rhizoplane bacterial communities (R^2 value) is explained by the growth stage, followed by the time point.

	R^2	F	p-value	Significance
Treatment (Control vs Zeb)	0.02418	4.3122	0.001	***
Age (Days)	0.10648	18.9896	0.001	***
Growth Stage	0.20613	2.2975	0.001	***
Treatment:Age	0.00872	1.5558	0.077	
Treatment:Growth Stage	0.06569	1.4644	0.002	**

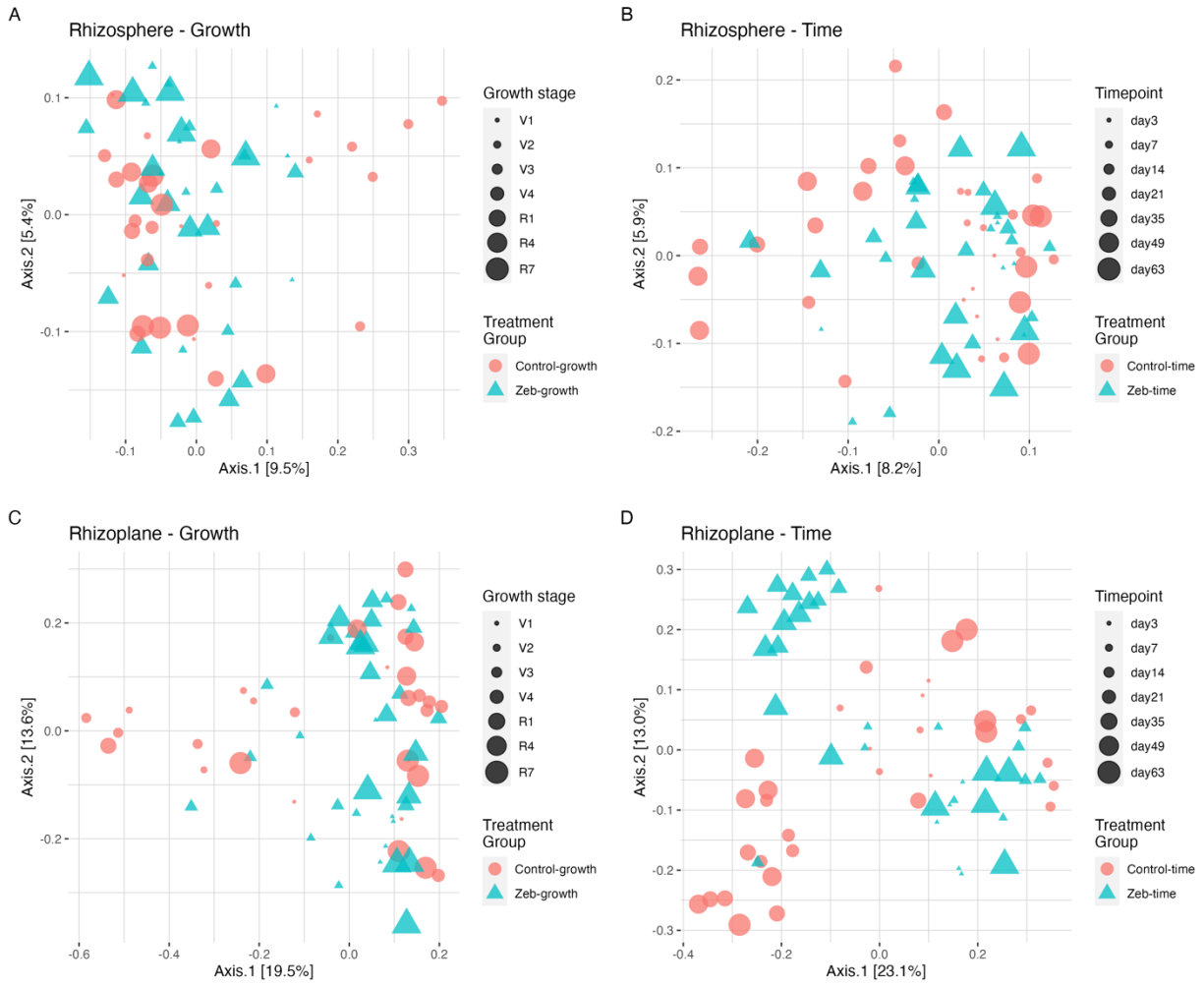

Figure 3.5. Beta diversity PCoA of the Bray-Curtis distances of the rhizosphere **(A, B)** and rhizoplane **(C,D)** microbial communities harvested at the Growth Stage (left) and Time Series (right) sampling points. Sampling point is indicated by symbol size, Treatment Groups are statistically different in all sampling series (PERMANOVA, A: r2=0.03035 F=2.2850 p=0.0001*, B: r2=0.02404 F=1.7894 p=0.0004*, C: r2=0.03567 F=2.9122 p=0.0015*, D: r2=0.03382 F=3.2968 p=0.0011*). When sampling points are compared between Control and Zeb within each ordination, the rhizosphere growth stage and time series samples are statistically different from each other at all but the earliest sample points at the V1 and V2 stages, and day 3 and day 7, respectively. In the rhizoplane, the growth stage samples are not statistically different at the V3, V4 and R7 stages, while all samples are statistically different at each time point in the time series (see supplementary table for sampling point statistics).

Rhizoplane communities are aligned in the growth stage series and contain selected taxa

To further investigate the similarities between the control and zeb samples in each sampling series, a Procrustes concordance analysis was performed to compare the structure of the PCoA ordinations. The Procrustes analysis revealed concordance between the rhizoplane control and zeb ordinations in the growth stage series, further confirming that the bacterial communities in these samples are aligned over the lifecycle of the plant, while all other ordinations were not concordant (Rhizoplane Growth: Procrustes Sum of Squares = 0.8591, Correlation in a symmetric Procrustes rotation = 0.3754, Significance value = 0.024*. Other significance values: Rhizosphere Growth = 0.332, Rhizosphere Time = 0.498, Rhizoplane Time = 0.989) (Figure 3.6). Additionally, when the 57 most abundant taxa in the ordination are plotted on a heatmap across all rhizoplane samples, the control and zeb samples mostly cluster together at the sampling points based on growth stage, while in the time series the control and zeb cluster together at only the earliest time points and appear to separate from each other at later time points (Figure 3.7A). This also supports the finding that there is greater similarity between treatments based on growth stage.

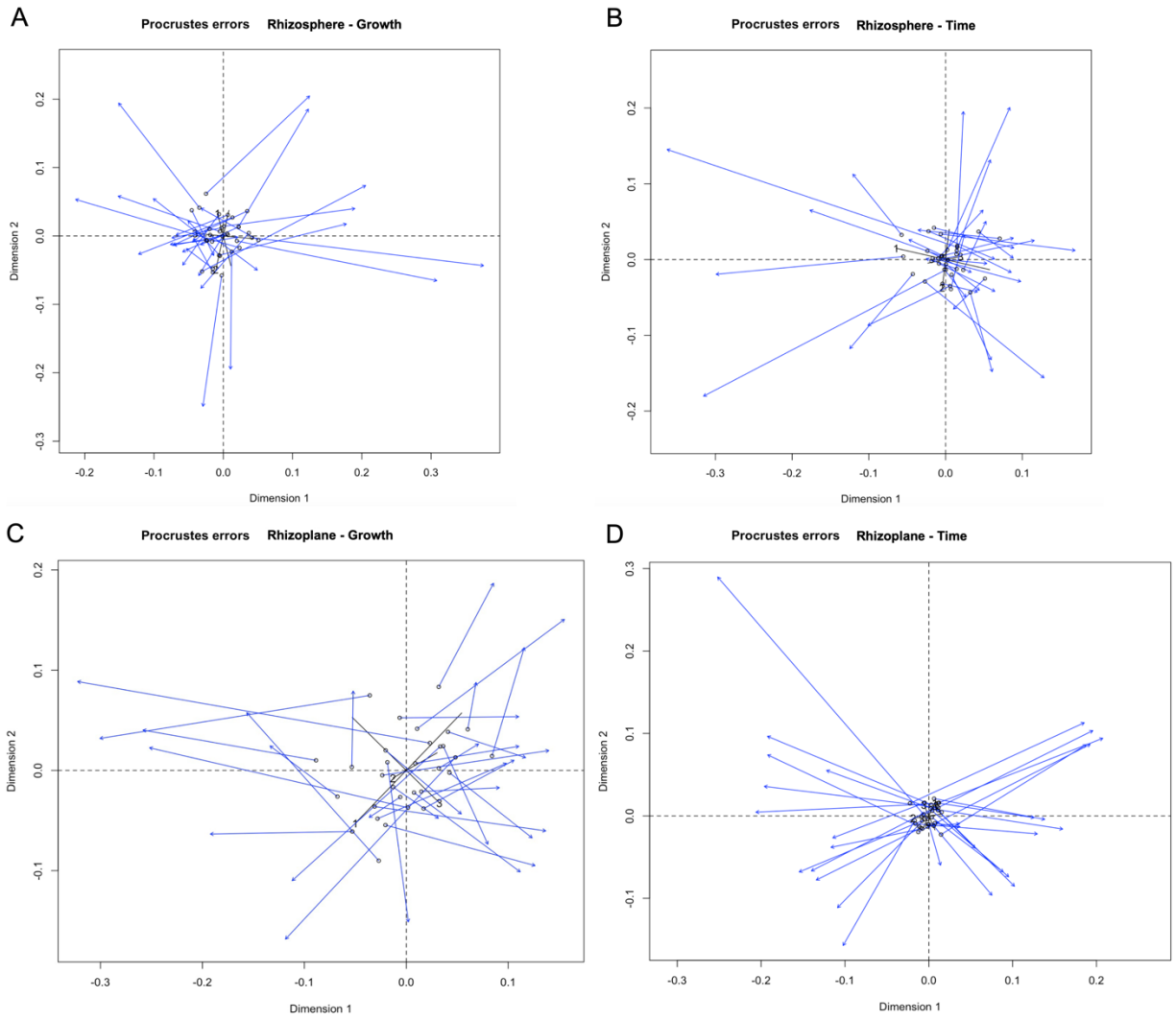

Figure 3.6. Procrustes concordance analysis between Control and Zeb samples in Bray-Curtis distance PCoA ordinations. **A.** Rhizosphere Growth Stage, **B.** Rhizosphere Time Series, **C.** Rhizoplane Growth Stage, **D.** Rhizoplane Time Series. Blue arrows indicate distances between correlating sample points between Control and Zeb. Only the Rhizoplane Growth Stage (C) control and zeb ordinations are concordant with each other (C: Procrustes Sum of Squares = 0.8591, Correlation in a symmetric Procrustes rotation = 0.3754, Significance value = 0.024*. Other significance values: A = 0.332, B = 0.498, C = 0.989).

In order to further investigate the assembly processes in the rhizoplane of common bean, the Sloan neutral model was used to investigate taxa that may be assembled by selection in the plant environment[54,55]. The Sloan neutral model was fit to the entire rhizoplane growth stage dataset, including both the control and zeb treatment groups. Of the ASVs in the

rhizoplane dataset, 13.6% were above prediction in the model, suggesting selection by the plant environment over the life cycle of the plant (Figure 3.7B). Alternatively, 4.3% were below prediction, suggesting that they are dispersal limited, while 82.1% of the taxa fell within the neutral model, which suggests they are assembled by neutral or stochastic processes (Figure 3.7B).

Additionally, because the growth stages had the greatest influence on the assembly of the rhizoplane bacterial communities in this study, indicator taxa were identified across the rhizoplane growth dataset based on growth stage. There were 204 ASVs identified as indicator taxa associated with the plant growth stages, 60 and 63 of which were associated with the late vegetative and early reproductive growth stages (V4 and R1), 33 were associated with the V1 stage, 26 were associated with R7, and few taxa were identified as indicators of the V2, V3 and R4 stages. When these taxa were investigated in the Sloan neutral model, 75 indicator taxa were above prediction (Figure 3.7B). Most of the indicator taxa that were selected by the plant are associated with the V1, V4 and R1 growth stages, while there were very few selected indicator taxa from other growth stages (Figure 3.7C). These selected indicator taxa were diverse and included ASVs from 29 bacterial classes, with over 40% of the selected taxa identified in the classes Alphaproteobacteria, Thermoleophilia, Bacilli and Gammaproteobacteria (Figure 3.7C). The selected ASVs included taxa from genera *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Pseudolabrys, Duganella, Paenibacillus, Bacillus, Thermoactinomyces* and *Gaiella*, and families Hyphomonadaceae, Sphingomonadaceae, and Nitrosomonadaceaeand and many others that are known to be commonly associated with plant microbiomes (See Table S3.2 for full taxonomy of selected

indicator taxa). Overall, these results confirm the significant influence of the plant growth stage on the resulting rhizoplane community, and provide evidence that the microbial communities are under selection by the plant.

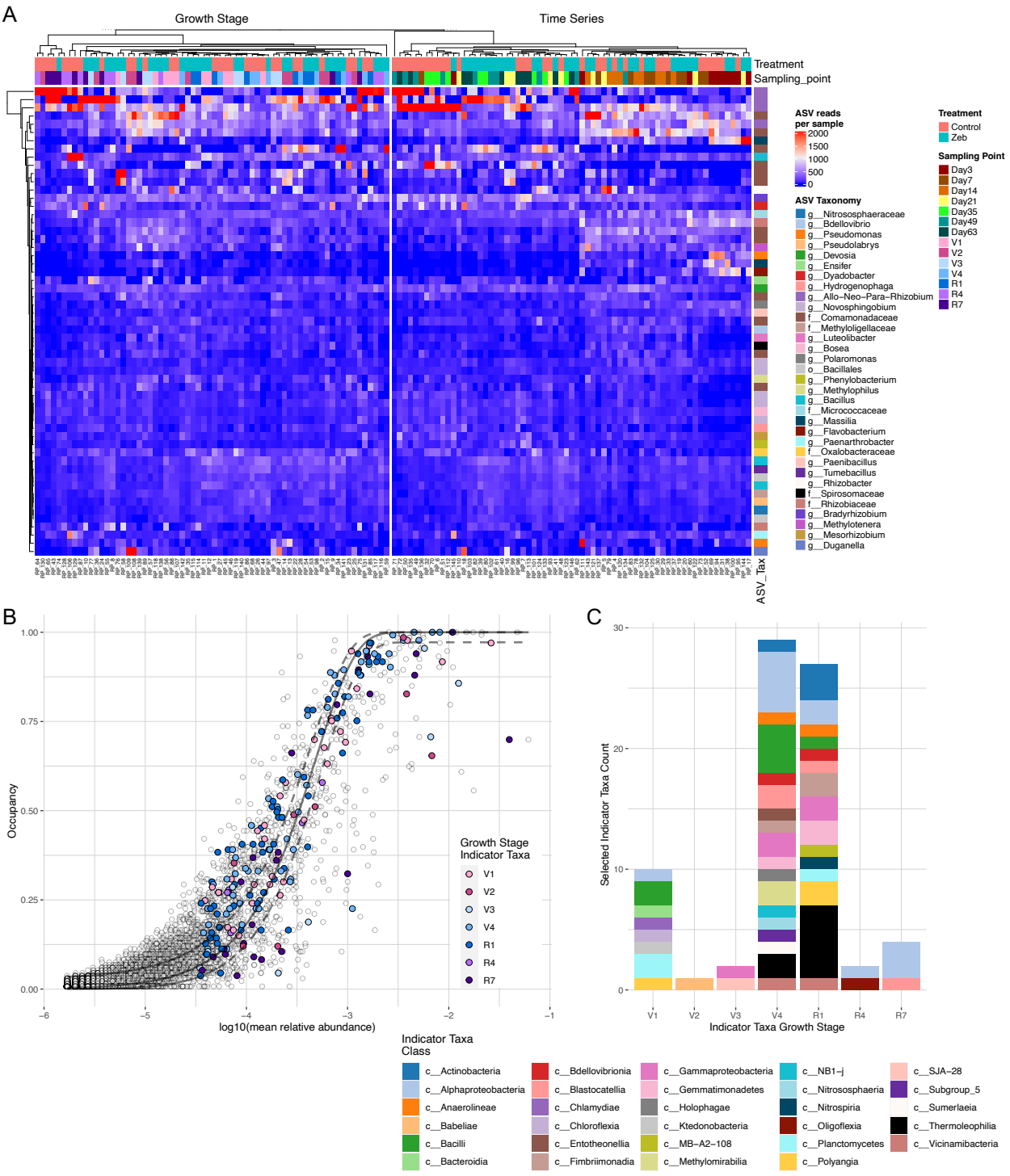

Figure 3.7. Analysis of bacterial communities in the rhizoplane. **A.** Heatmap of the 57 most abundant ASVs (rows) in the rhizoplane samples (columns) across the Growth Stage and Time Series sampling schemes. Treatment bar indicates Control or Zeb treated samples. Sampling point bar indicates which sampling point the sample was taken at. Colored column indicates the most resolved taxonomic identity of the ASV. **B.** Abundance-occupancy distribution of ASVs in the rhizoplane samples. N=9,569 ASVs. Solid and dashed lines represent the fit of the neutral

Figure 3.7 (cont'd)
model and 95% confidence interval. Colored points represent ASVs identified as indicator taxa in the rhizoplane at each plant growth stage. **C.** Indicator taxa ASVs that fall above the prediction of the neutral model, grouped by which plant growth stage they are associated with. Colors indicate the taxonomic identification for the ASV at the Class level.

DISCUSSION

The goal of this study was to elucidate the proportion of the influence of plant growth stage verses time in driving the assembly of the root microbiome over the common bean life cycle. There has been a great deal of work done on the assembly of the rhizoplane and rhizosphere over the life cycle of the plant, and many studies have documented the influence of plant phytohormones and chemical feedbacks on the development of the rhizosphere[22,23,28,56]. However, it has been difficult to assess the influence of plant growth stage and growing time separately in the root microbiome because these variables are tightly linked. This study utilized a growth delay caused by zebularine to study the development of the bean microbiome on two different sampling series in order to separate the variables of growth and time.

We found that the rhizoplane bacterial community is highly responsive to plant growth stage regardless of plant age in the soil, while the rhizosphere soils are less significantly impacted by the plant which has been observed in previous studies[27,57]. Rhizoplane communities in the zebularine treated plants were closely aligned to the control plants based on growth stage, despite being harvested weeks apart at times. The beta-diversity of the rhizoplane communities was similar between control and zeb treated plants across the growth stage sampling points, and the rhizoplane growth stage ordinations were concordant in their structures. The influence of plant growth stage on the rhizoplane communities in our study accounted for 20% of the variation in the communities, while time accounted for 10%. A

considerable amount of the variation in the communities is unexplained by the variables in this study, which aligns with our observation that a large proportion of taxa in the rhizoplane were within the prediction of the neutral model and assembled by stochastic processes. Stochastic assembly processes have also been observed in other studies investigating the development of the plant microbiome, such as in canola, poplar and soybean(bell 2022, dove 2021, moroenyane 2021)[18,26,58]. We also identified many indicator taxa linked to growth stage in the common bean rhizoplane that are under selection by the plant, and found that the bacterial communities in the late vegetative and early reproductive stages were associated with the highest number of indicator taxa.

Understanding the influence of the plant on the microbiome is not only important from an ecological standpoint, but also has significant implications for the growing field of microbiome engineering in agricultural crops[59,60]. Developing methods to identify plant growth-promoting bacteria that could be applied to plants in the field is a rapidly growing area of research. It can be difficult to isolate beneficial bacteria from plant microbiomes, and bacteria used in agricultural applications are not always isolated from the crop they are being applied to[61–63]. Our study proposes a potential framework for identifying bacterial targets for microbial applications utilizing the Sloan neutral model. Identifying indicator taxa that are under selection by the plant in various species may be a promising starting point in developing crop-specific microbial treatments for use in the field [64–67]. Additionally, since a majority of the indicator taxa under selection in this study were from the V1, V4 and R1 growth stages, this may be a potential area for further studies to investigate if there is a greater selective pressure by the plant at these growth stages. When biological products are being developed for use in the field,

researchers should consider the timing of the application of these microbial species, as the growth stage of the plant may be an important factor in maximizing the effect of the product, but more work is needed in this area.

In addition to the timing of microbial applications being a potential consideration for plant microbiome applications, the bacterial species being applied is also important for effective microbial products. The indicator ASVs identified that were under selection in the rhizoplane were highly diverse, but contained many taxa that are known to have plant growth-promoting properties or are commonly associated with plant microbiomes, including many ASVs from the classes Alphaproteobacteria, Thermoleophilia, Bacilli and Gammaproteobacteria[68–75]. These taxa that are being selected at in the rhizoplane environment should be investigated for potential isolation and use in microbial inocula in common bean, and similar experiments investigating selected taxa could be used to identify important microbiome members in other crops as well.

In summary, this study confirms the significant effect of plant growth on the development and assembly of the rhizoplane microbiome in common bean, and reveals the selective influence of plant growth stage over the plant life cycle. While time does have an independent influence on the bacterial communities in the rhizoplane in common bean, a greater proportion of the rhizoplane variation was influenced by plant growth stage. Additionally, a large proportion of the variation in the communities was unexplained by the variables investigated, and the majority of the ASVs were within the prediction of the Sloan neutral model. Indicator taxa associated with the late vegetative and early reproductive stages were under selection by the plant and should be investigated for use to improve crop yields in

the field. Our results suggest that plant growth stage may be an important factor to consider when developing products for microbiome modification, and further work should investigate the selective influence of the plant over time. Overall, this works sheds light on the importance of plant growth stage on the development of the rhizoplane microbiome, and provides a framework for further identification of plant growth-promoting bacteria for agricultural applications.

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APPENDIX C:
CHAPTER 4 SUPPLEMENTARY MATERIALS

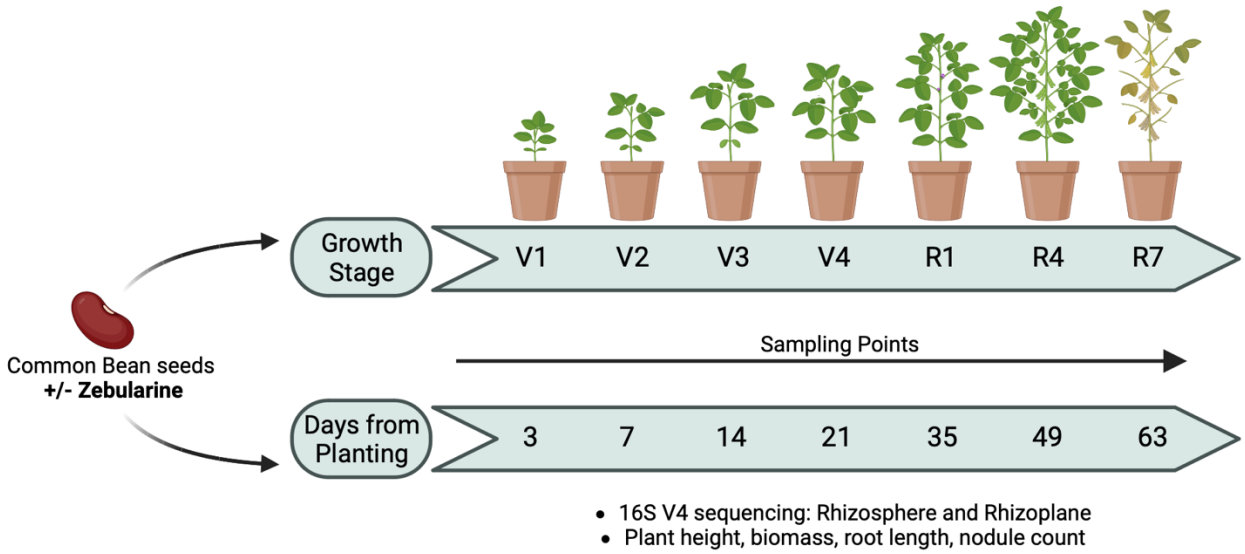

Figure S3.1. Experimental Design. Seeds were treated with and without 5mM zebularine, then grown over two sampling schemes based on plant growth stage or days since planting.

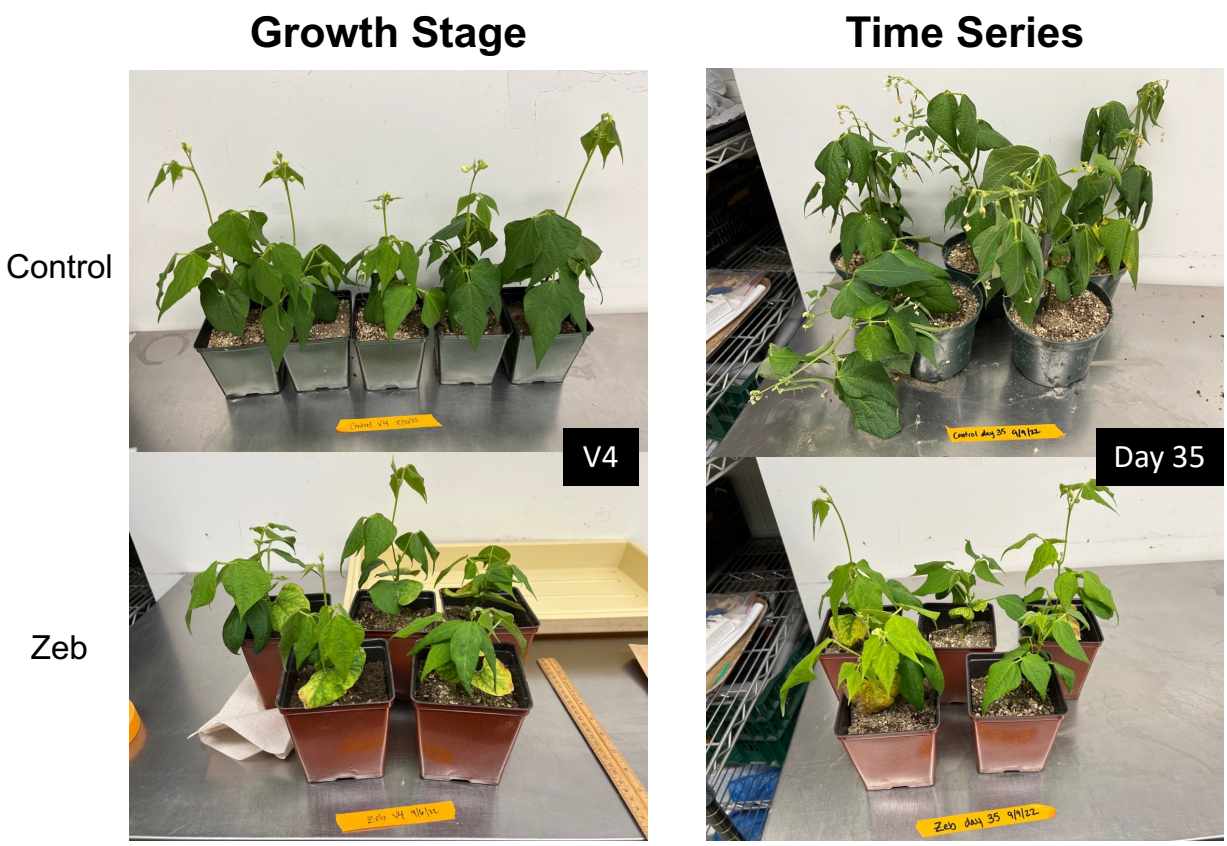

Figure S3.2. Comparison of plant above-ground growth in control (top) and zebularine treated (bottom) plants in the Growth Stage series at the V4 stage, and in the Time Series 35 days after planting.

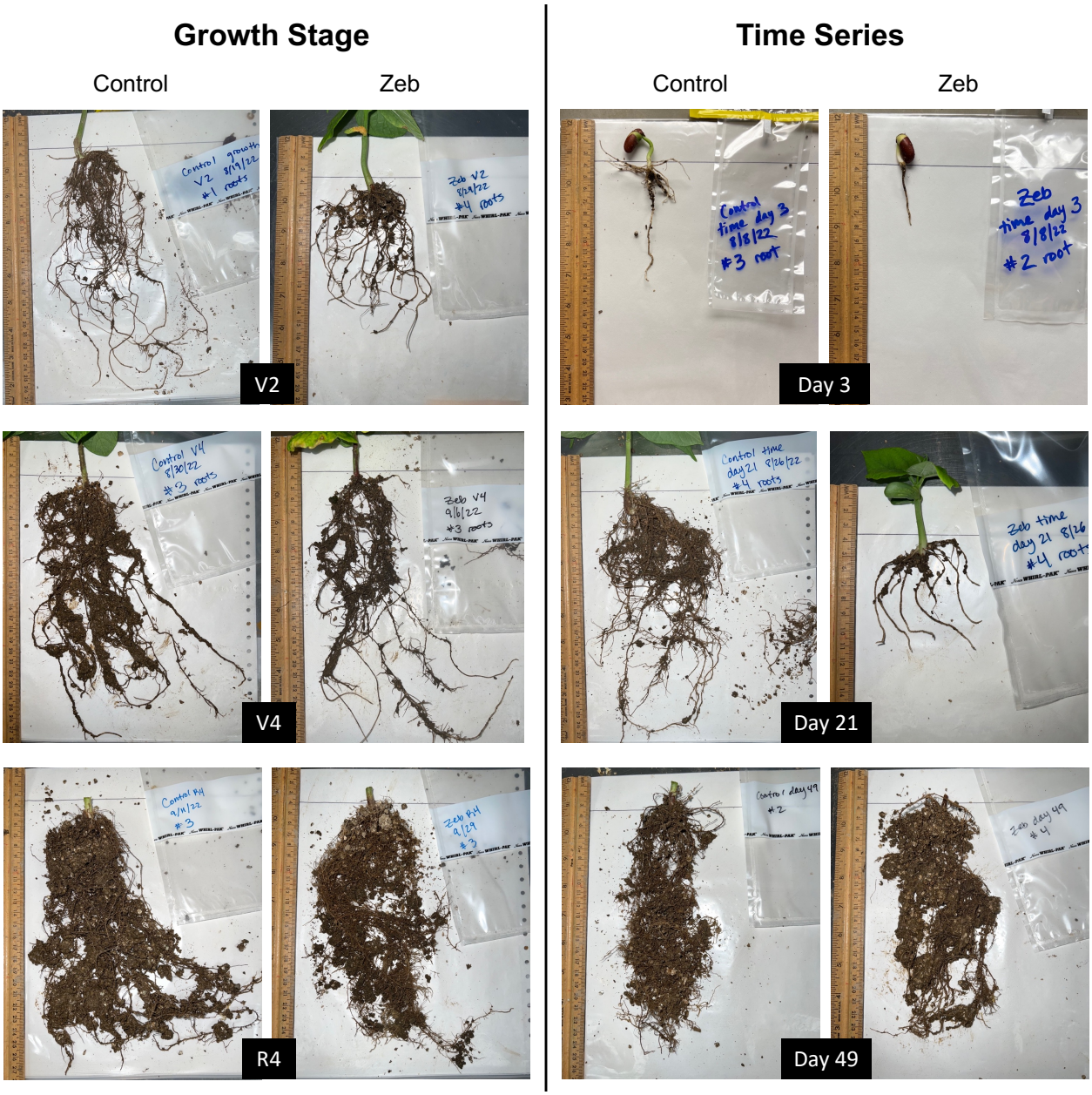

Figure S3.3. Comparison of plant root growth in control and zebularine treated plants in the Growth Stage series (left) at the V2, V4 and R4 stages, and in the Time Series (right) 3, 21 and 49 days after planting.

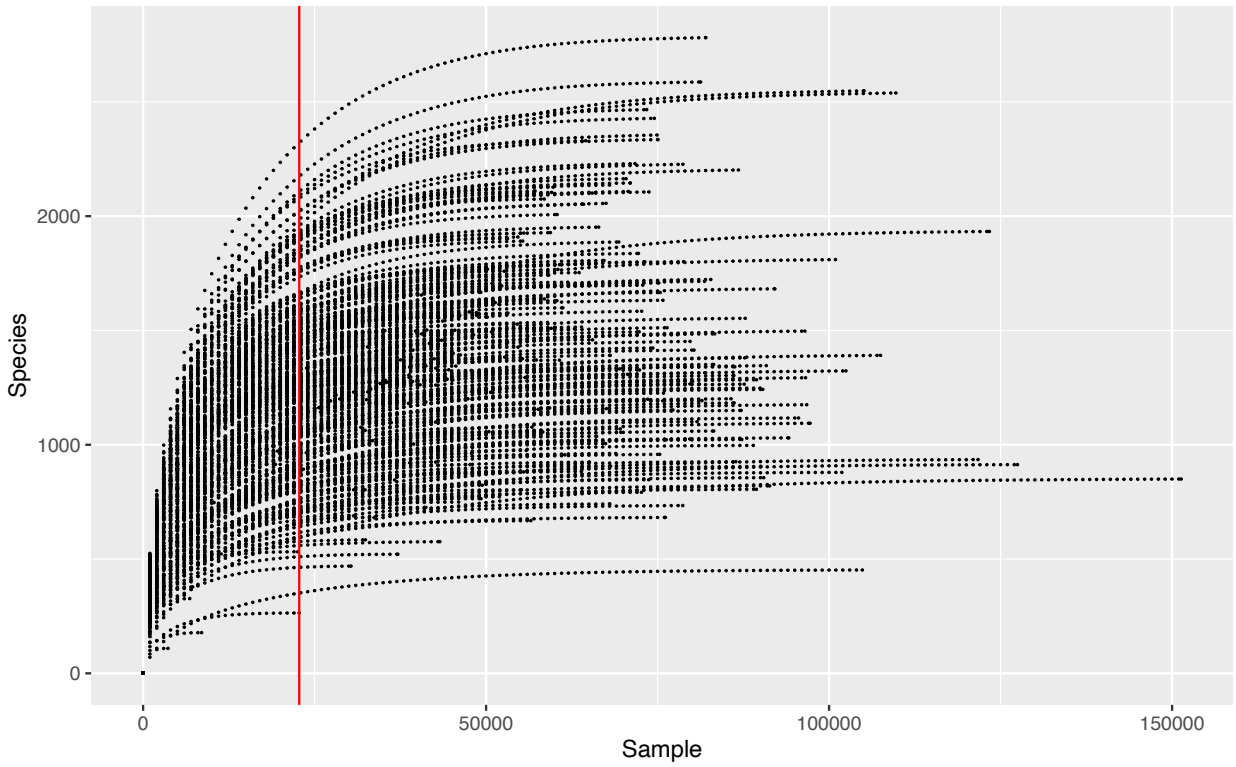

Figure S3.4. Rarefaction curves of combined rhizosphere and rhizoplane datasets after sequence decontamination. Samples were rarefied to 22,761 reads (red, vertical line). 9 samples were removed that did not meet the minimum DNA read threshold.

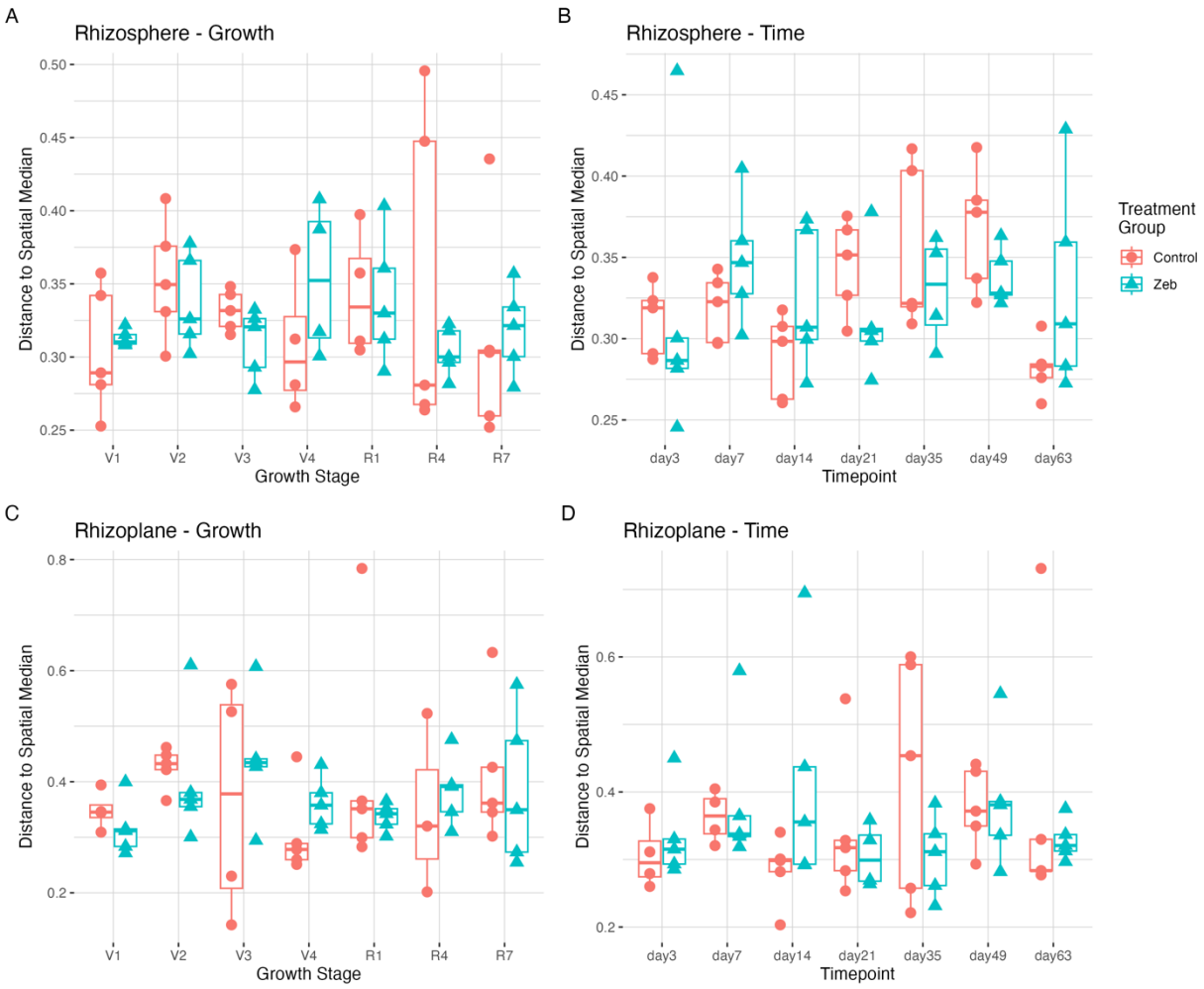

Figure S3.5. Beta-dispersion, the distance to the spatial median of sample groups, in Bray-Curtis PCoA ordinations. **A.** Rhizosphere Growth Stage, **B.** Rhizosphere Time Series, **C.** Rhizoplane Growth Stage, **D.** Rhizoplane Time Series. There are no significant differences in dispersion between the Control and Zeb treatments at any sampling point in either root compartment.

Table S3.1

Bray-Curtis PERMANOVA and pairwise results

	Df	SumOfSqs	R2	F	Pr(>F)	
PERMANOVA: Rhizosphere, Control Growth vs Control Time						
group	1	0.158	0.01909	1.2846	0.0697	
PERMANOVA: Rhizosphere, Zeb Growth vs Zeb Time						
group	1	0.1491	0.01917	1.2897	0.038	*
PERMANOVA: Rhizoplane, Control Growth vs Control Time						
group	1		0.02354	1.4944	0.1142	
PERMANOVA: Rhizoplane, Zeb Growth vs Zeb Time						
group	1	0.383	0.03051	2.1087	0.0188	*
PERMANOVA: Rhizoplane, Growth Stage series, Control vs Zeb						
treatment	1	0.4388	0.03567	2.9122	0.0015	**
stage	6	2.7742	0.22551	3.0686	0.0001	***
treatment:stage	6	1.2538	0.10192	1.3868	0.0128	*
Pairwise test: Rhizoplane, Control vs. Zeb at growth stages						
Zeb_growth_V1_vs_Control_growth_V1						
group_stage	1	0.20092	0.18031	1.7598	0.0161	*
Zeb_growth_V2_vs_Control_growth_V2						
group_stage	1	0.25737	0.17024	1.6413	0.0172	*
Control_growth_V3_vs_Zeb_growth_V3						
group_stage	1	0.1877	0.17949	1.3125	0.3031	
Control_growth_V4_vs_Zeb_growth_V4						
group_stage	1	0.18781	0.12141	1.1055	0.3406	
Control_growth_R1_vs_Zeb_growth_R1						
group_stage	1	0.19062	0.198	1.7282	0.0401	*
Zeb_growth_R4_vs_Control_growth_R4						
group_stage	1	0.34333	0.19559	1.9452	0.0159	*

Table S3.1 (cont'd)

Zeb_growth_R7_vs_Control_growth_R7						
group_stage	1	0.32125	0.2024	1.7763	0.0726	
PERMANOVA: Rhizoplane, Time series, Control vs Zeb						
treatment	1	0.4435	0.03382	3.2968	0.0011	**
stage	6	3.7329	0.28468	4.6248	0.0001	***
treatment:stage	6	1.8065	0.13777	2.2382	0.0001	***
Pairwise test: Rhizoplane, Control vs. Zeb at days since planting						
Zeb_time_day3_vs_Control_time_day3						
group_stage	1	0.20158	0.15675	1.4871	0.0148	*
Zeb_time_day7_vs_Control_time_day7						
group_stage	1	0.24231	0.17966	1.7521	0.0391	*
Control_time_day14_vs_Zeb_time_day14						
group_stage	1	0.24532	0.24769	2.3047	0.0162	*
Zeb_time_day21_vs_Control_time_day21						
group_stage	1	0.21636	0.17412	1.4758	0.0318	*
Control_time_day35_vs_Zeb_time_day35						
group_stage	1	0.43207	0.35432	3.8413	0.015	*
Control_time_day49_vs_Zeb_time_day49						
group_stage	1	0.57954	0.32921	3.9261	0.0085	**
Zeb_time_day63_vs_Control_time_day63						
group_stage	1	0.32498	0.21318	2.1675	0.0081	**
PERMANOVA: Rhizosphere, Growth Stage series, Control vs Zeb						
treatment	1	0.2474	0.03035	2.285	1.00E-04	***
stage	6	1.1804	0.14481	1.8168	1.00E-04	***
treatment:stage	6	0.9847	0.1208	1.5157	1.00E-04	***
Pairwise test: Rhizosphere, Control vs. Zeb at growth stages						
Zeb_growth_V1_vs_Control_growth_V1						
group_stage	1	0.13643	0.16989	1.2279	0.1115	

Table S3.1 (cont'd)

Control_growth_V2_vs_Zeb_growth_V2						
group_stage	1	0.12958	0.13639	1.1055	0.2226	
Control_growth_V3_vs_Zeb_growth_V3						
group_stage	1	0.20019	0.18365	1.7998	0.0092	**
Control_growth_V4_vs_Zeb_growth_V4						
group_stage	1	0.16125	0.16639	1.5968	0.0077	**
Control_growth_R1_vs_Zeb_growth_R1						
group_stage	1	0.16426	0.17631	1.7124	0.0067	**
Zeb_growth_R4_vs_Control_growth_R4						
group_stage	1	0.17196	0.15186	1.4324	0.018	*
Zeb_growth_R7_vs_Control_growth_R7						
group_stage	1	0.26716	0.24428	2.5859	0.0084	**
PERMANOVA: Rhizosphere, Time series, Control vs Zeb						
treatment	1	0.1932	0.02404	1.7894	4.00E-04	***
stage	6	1.095	0.13619	1.6899	1.00E-04	***
treatment:stage	6	0.8122	0.10102	1.2535	4.00E-04	***
Pairwise test: Rhizosphere, Control vs. Zeb at days since planting						
Zeb_time_day3_vs_Control_time_day3						
group_stage	1	0.10228	0.11854	1.0758	0.324	
Zeb_time_day7_vs_Control_time_day7						
group_stage	1	0.08906	0.10415	0.93	0.7113	
Control_time_day14_vs_Zeb_time_day14						
group_stage	1	0.12559	0.13481	1.2466	0.0298	*
Control_time_day21_vs_Zeb_time_day21						
group_stage	1	0.15609	0.14827	1.3926	0.0069	**
Control_time_day35_vs_Zeb_time_day35						
group_stage	1	0.16751	0.16108	1.5361	0.0083	**

Table S3.1 (cont'd)

Control_time_day49_vs_Zeb_time_day49						
group_stage	1	0.16252	0.1627	1.3602	0.0423	*
Control_time_day63_vs_Zeb_time_day63						
group_stage	1	0.19904	0.16579	1.5899	0.0086	**

Table S3.2 Supplemental File: Table of selected indicator taxa

Table S3.3 Supplemental File: Metadata table of DNA extractions

CHAPTER 5:

Conclusions and future directions

This work contributes important knowledge to the area of plant microbiome research, particularly in understanding the microbiome of common bean plants and how they are impacted by environmental stress. Changing climate conditions will continue to pose a threat to agricultural crops through impacts such as warming, drought, and extreme weather events, and improving the resilience of plants to these impacts is becoming increasingly important[1,2]. This is especially true for staple food crops like common bean, as many people depend on beans for their livelihood and survival in areas that are at particular risk for climate change impacts[3,4]. Plant-microbe interactions are also at risk of the negative effects of climate change, and it is important to understand how these host-microbe associations are changing to allow researchers to better support crops into the future[5–7].

Throughout this research, we examined the impacts of repeated drought exposure on common been root and rhizosphere communities, the vertical transmission of seed microbiomes under abiotic treatment, and the development of the rhizosphere communities over the bean life cycle. In chapter 2, the Red Hawk plants grown in different soils and the different plant genotypes were found to have significantly different root and rhizosphere microbiomes in alpha and beta diversity measures. The drought stress treatments had little effect on the bacterial communities of the Red Hawk plants, but Flavert plant microbiomes were significantly impacted by the drought and showed evidence of legacy effects of multigenerational exposure. These results suggest that plant genotype may play a role in responses to environmental stress, and the microbiome of the Red Hawk variety may be stable under drought stress. Microbiome modification to support Red Hawk plants under drought may not be effective, and other solutions to climate challenges should be investigated.

In chapter 3, we investigated the vertical transmission of bacterial taxa through the Red Hawk bean seeds over two generations of abiotic treatment with drought stress or increased nutrient concentration. The abiotic treatments had little effect on the seed microbiome, with the only evidence of a treatment effect in the beta diversity of second-generation seeds after the Generation 1 parent had been exposed to drought stress. However, a stable signature of the seed microbiome was observed over multiple generations, with 22 bacterial ASVs identified as highly prevalent in the seeds of all three generations, despite differences in soil and treatment conditions. The transmitted seed endophytes in our study also contained known bacterial taxa that are part of the common bean core microbiome. In summary, we identified a stable and consistent seed microbiome in Red Hawk seeds with strong evidence of vertical transmission, and these seed taxa should be investigated further to understand their role in plant health.

Lastly, in chapter 4, we explored the separate influences of plant growth stage and time on the assembly of the rhizosphere and rhizoplane microbiome in common bean Red Hawk plants. Through an innovative experimental design using zebularine treatment to cause a growth delay in the Red Hawk plants, we discovered that plant growth stage is the most significant driver of the rhizoplane community assembly. The control and zebularine treated plants had similar rhizoplane bacterial communities despite being harvested on different dates, while the rhizosphere soils were less directly affected by the plant. Plant growth stage accounted for 20% of the variation in the rhizoplane communities, while time accounted for 10%. A large proportion of the bacterial community variation was unexplained in the dataset, and investigating the communities with the Sloan neutral model[8] revealed that much of the

bacterial communities are controlled by neutral or stochastic processes in this study. Indicator taxa associated with the plant across growth stages were identified and were found to be under selection by the plant environment in the rhizoplane. These ASVs should be investigated further as potential targets for beneficial bacterial applications in common bean.

Future work based on this research could include many different avenues of investigation. Each of these experiments identified potentially beneficial plant microbiome members that should be investigated further and uncovered potential frameworks for further identification of important microbiome members for use in agricultural applications. One area that could be investigated is the consistently transmitted ASVs identified in the seed microbiome study. These taxa are consistently associated with the seeds over multiple generations despite differences in soil and abiotic treatment. The taxa identified in the drought and rhizosphere assembly studies should be compared to the prevalent ASVs in the seed study. This may elucidate the role of these ASVs in other plant microbiome compartments and their potential benefits to the plant under varying environmental conditions. Further, the ASVs identified in this work, both in the seed microbiome and the indicator taxa that were under selection in the rhizoplane, could be targeted for isolation from common bean seeds in order to assess their potential benefits *in vivo*[9]. Isolating plant microbiome members has proved challenging in past work, but having specific targets for isolation may help create new methods to isolate these taxa from plant tissues[10–12]. Once bacteria are isolated from the plant, this opens many doors for plant growth studies to elucidate which isolates are most beneficial and what their mechanisms are in supporting the plant. There has been work done utilizing microbial inoculants to improve plant performance in controlled growth studies with promising

results and it would be particularly interesting to apply the prevalent seed ASVs and rhizoplane indicator taxa to plants to study their effects on plant growth[13–15].

Additionally, this work provides a framework for understanding how the pant root microbiome is influenced by the plant over time. Analyzing plant microbiomes over the life cycle of the plant has elucidated a clear impact of growth stage on the development of the rhizoplane in common bean. Utilizing the neutral model to assess the selection of indicator taxa by the plant environment has also further advanced our knowledge of the assembly processes in the rhizoplane ecosystem. Future studies could investigate the selective influence of the plant over time at each growth stage, verses broadly as we investigated here, which could provide insight into potential microbial applications in the field. Knowing when beneficial microbes are most important for the plant over its growth stages, and how susceptible the root microbiome is to microbial invasion at these stages, are important knowledge gaps that should be investigated to advance the field of microbiome engineering[16–19]. This experimental design could be applied in various agricultural crops in order to identify key bacterial taxa and growth stages in the plant microbiome. [16–19]

Lastly, it is important to consider the applicability of these small-scale studies to plants growing in the field. Varying soil types and geographic locations, environmental and disease conditions, and management practices can all have an impact on the plant microbiome. It is important when studying the plant microbiome to consider these variables that could affect the plant in real-world conditions[20] . Our drought study partially addresses these challenges by studying the effects of treatment in different plant genotypes and soils. As researchers continue to develop microbial strategies for supporting crops in the field, it will become increasingly

important to understand how different plant varieties respond to microbial treatments and how environmental factors can alter the effectiveness of these products. Additionally, it is important to move research from controlled conditions to the field at larger scales with greater replication, in order to understand if the same patterns are evident in both environments.

Overall, this work provides important knowledge to the field of plant microbiome research, particularly in the potential of the plant microbiome to support plants under stress conditions, the stable transmission of seed microbiome taxa, and the assembly of the rhizoplane over the life of the plant. Understanding this basic science of the common bean microbiome is important to move the field forward and will help researchers find sustainable solutions for the future of agriculture. The need to support agricultural crops under changing climate conditions will become increasingly urgent as the global population continues to rise, and plant microbiome engineering may hold the key to improving the resilience of agricultural systems[21]. This research on the common bean microbiome will provide tools and targets for future studies in beneficial bacteria for sustainable agricultural solutions.

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